Genetic Variabilities of *Stevia rebaudiana* Bertoni Cultivated in Malaysia as Revealed by Morphological, Chemical and Molecular Characterisations

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**ARTICLE INFO**

*Keywords:* Genetic Variabilities, ISSR Marker, Morphology, *Stevia rebaudiana*, Stevial glycosides

**Article History:**
Received: March 28, 2017
Accepted: March 19, 2018

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**ABSTRACT**

*Stevia rebaudiana* Bertoni originally from Paraguay belongs in Asteraceae family. It is an alternative source of non-caloric sweetener due to the sweet steviol glycosides contained in the leaves. As an introduced species in Malaysia, it is important to elucidate the genetic variabilities and relatedness among stevia accessions in order to broaden the genetic basis for future stevia breeding. This study described morphological and chemical variations and investigates genetic relationships among stevia accessions derived from across Malaysia and Paraguay using inter simple sequence repeats (ISSR) markers. HPLC (high-performance liquid chromatography) analysis also revealed high variability with stevioside content between 4.54 % (Taman Pertanian) to 20.36 % (Bangi) and rebaudioside A content varied between 0.3 % (Nilai) to 2.04 % (MNQ). From 32 ISSR markers, a total of 323 bands were scored, of which 264 (78 %) were polymorphic. The dendrogram from UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis separated 17 stevia accessions into 3 main groups. Rawang and Nilai were found to be closely related. The wide genetic variabilities among stevia accessions are a promising indicator towards the development of new stevia varieties. This valuable information will be able to assist parental selection in future stevia breeding programmes.

ISSN: 0126-0537 Accredited by DIKTI Decree No: 60/E/KPT/2016

Cite this as: Othman, H. S., Osman, M., & Zainuddin, Z. (2018). Genetic variabilities of *Stevia rebaudiana* Bertoni cultivated in Malaysia as revealed by morphological, chemical and molecular characterisations. AGRIVITA Journal of Agricultural Science, 40(2), 267–283. http://doi.org/10.17503/agrivita.v40i2.1365

**INTRODUCTION**

Stevia from the family Asteraceae is a small, dense, shrubby perennial plant that can grow 50 cm to 1 m in height when cultivated. The leaves are intense green with lanceolate shape growing diametrically opposite on the stems. Trichome on the leaf surface has 2 distinct sizes; 1 large (4-5 µm) and 1 small (2.5 µm) (Yadav, Singh, Dhyani, & Ahuja, 2011). Stevia flowers are normally small (7-15 mm), white and irregular cyme arrangement. The flower is perfect (hermaphrodite) having both male and female organs, contained in tiny corymbbs with two or six florets (Goettemoeller & Ching, 1999). Seeds are contained in slender achenes about 3 mm in length with each achene has about 20 persistent pappus bristle cymes (Yadav, Singh, Dhyani, & Ahuja, 2011). The roots are fibrous and dense. Naturally, being a short-day species, it requires about 13 hours of critical day length (Ceunen, Werbrouck, & Geuns, 2012; Pereira, Storck, Lopes, Martin, & Bisognin, 2016) and flowering can be initiated after a minimum of four true leaves production (Yadav, Singh, Dhyani, & Ahuja, 2011).

Stevia is mainly grown for its leaves because the leaves produce steviolbioside, stevioside, rebaudioside A–F, rebaudioside M, dulcoside A and rubusoside (Madan et al., 2010; Pal et al., 2015; Prakash, Markosyan, & Bunders, 2014; Steinmetz & Lin, 2009). Stevioside is reported to be the major glycoside and it has been extensively used as sweetener particularly in Japan and other parts of the world. Unlike stevioside, rebaudioside A is sweeter and more palatable than stevioside which has become the main interest for breeders to produce varieties with the improved content of rebaudioside A (Rajasekaran, Giridhar, & Ravishankar, 2007; Yadav, Singh, Dhyani, & Ahuja, 2011). The relative sweetness of stevioside is about 110-270 times sweeter, while rebaudioside A is 150-320 times sweeter.
sweetener from stevia has been great interest in stevia breeding and production (Yadav, Singh, Dhyani, & Ahuja, 2011). This natural high potency sweetener from stevia has been great interest in stevia breeding and production (Yadav, Singh, Dhyani, & Ahuja, 2011).

The first commercial cultivation of stevia in Paraguay started around 1964 (Madan et al., 2010). However, in Malaysia, stevia cultivation dates back to the early 1970’s (Tan, Ghawas, Najib, & Zawayi, 2008). Stevia cultivation has become an interest in Malaysia as a result of the sugar shortage experienced by the country. Malaysia does not have the optimum climate to enhance sugar cane cultivation; hence the local demand for sugar is highly dependent on sugar imports. Stevia plant is in high demand because of its potential as a non-caloric natural sweetener specifically towards individuals with body weight issues as well as diabetics (Mehta et al., 2011; Mogra & Dashora, 2009).

The current global mass production of stevia in Asia is China which produces more than 80 % of stevia. About 90 % of this export goes to Japan for natural sweeteners production where 40 % of the sweeteners in Japan is stevia-based (Chotikadachanarong & Dheeranupattana, 2013). Stevia research in countries like Japan, China, Russia, Korea and Taiwan has successfully released new and improved stevia varieties in terms of leaf yield and glycosides content (Yadav, Singh, Dhyani, & Ahuja, 2011). However, to date, there is no variety that is practical and suitable for Malaysian production in terms of leaf yield and glycosides content. There was a very limited stevia research that focuses on the germplasm characterization, apart from a work by 1990 and a preliminary report on evaluation and selection of stevia cultivated under Malaysian condition (Tan, Ghawas, Najib, & Zawayi, 2008). Thus, there has yet to be a conclusive study conducted on stevia cultivated in Malaysia that reports the morphological, chemical and molecular characterizations.

Traditionally, the characterization of a species is based on morphological characteristics. Since stevia collection in Malaysia has been acquired from different origins, their relatedness and genetic diversity is unknown. Hence, it is crucial to understand the stevia’s genetic diversity as this will help in the selection of diverse parents for hybridization and breeding for improved stevia. Selection of diverse parents is expected to generate a broader spectrum of variability among stevia progenies produced. The information on genetic diversity can be achieved through molecular approach using DNA markers; which are more stable, informative and reliable. Amongst the various DNA-based markers available, inter simple sequence repeats (ISSR) has shown to be among the simplest, most rapid and cheapest technique available (Bajpai et al., 2014). ISSR also gives high reproducibility (Gupta et al., 2008; Martins-Lopes et al., 2007) and polymorphism (Gupta et al., 2008; Martins-Lopes et al., 2007). Thus, the main aim of the present study was to conduct morphological, chemical and molecular characterizations of the collected stevia accessions. The information will be useful to assist parental selections of superior individuals for the use in future stevia breeding programme.

MATERIALS AND METHODS

Plant Material

The experiment was conducted at the experimental plot of Kulliyyah of Science, International Islamic University Malaysia Kuantan Pahang Malaysia from December 2013 to December 2014. A total of 17 stevia accessions were used in the present study. Fourteen stevia accessions were collected from different localities in all across Malaysia while 3 new introductions were from Paraguay (Table 1).

Morphological Parameters

All stevia accessions were prepared through stem micro-cutting propagation. The cuttings were grown in the regular nursery tray and when the plants were about 5 weeks old, they were transplanted into polythene bags to the experimental field of Kulliyyah of Science, International Islamic University Malaysia, Kuantan Pahang Malaysia. The field replicated trial was in a complete randomized design consisting of 20 plants per accessions and placed in a net house. The plants were irrigated twice daily and left to receive the normal sunlight for a duration of 12 hours which is typical for Malaysia climate. All stevia accessions used in the study were evaluated in terms of their morphology involving both qualitative and quantitative analysis. Qualitative morphological observation on the basis of leaf morphological variations was done as per the standard methodology (Balcázar-Vargas, Peñuela-Mora, van Andel, & Zuidema, 2012; Owens, Cieslak, Hart, Classen-Bockhoff, & Prusinkiewicz, 2016) which characterized the plant type and leaf characteristics that involved leaf shape, leaf tip, leaf base and leaf margin.
For data collection purposes, when the plants were at 2 months of age, the stems were pruned until about 2 cm above ground level. After 2 months of pruning, data collection was conducted. The observed parameters for quantitative morphological evaluations include 6 morphological characters namely: (i) plant height, (ii) days to flowering (iii) stem numbers, (iv) leaves number, (v) leaf size (length x width) and (vi) stem girth. Plant height and leaf size were measured with a ruler. The stem girth was measured using inelastic thread and curled around the base of the stem at a region above the soil level. The number of stems and leaves were counted visually. Data were analysed using SAS software version 8.02 (SAS Institute Inc., 1999) and subjected to one-way ANOVA and Tukey’s test at p = 0.05, where individual means for a particular trait was compared to the population mean of such character.

**Sweet Diterpene Glycosides Extraction**

Dry powdered leaves sample (0.2 g) was weighed and dissolved in 20 ml HPLC-grade water. The sample was put in a shaker water bath at 70 °C for 24 hours. The mixture was filtered by using vacuum filter to separate the extracts from the leaves residues. The crude extract was then stored in the chiller at 4 °C until further used. Prior to injection, an adequate volume of the crude extract (ca. 3 ml) was passed through a 1.2 µm nylon membrane filter (Merck Millipore, Darmstadt, Germany).

**HPLC analysis**

Standards stevioside and rebaudioside A were prepared separately in respective serial dilutions. All HPC analyses were conducted on Perkin Elmer FLEXAR™ liquid chromatograph system consisting of Flexar isocratic LC pump equipped with dual diode array detector and computerized data station using Chromera software. An NH-2 column (5 µm, 150 mm x 2 mm²) from Knauer (Germany) was used as the stationary phase and the temperature was maintained at 30 °C. The mobile phase consisted of a mixture of water and acetonitrile at 20:80 (v/v). The flow rate was maintained at 1.0 ml per minute. The total run time was 10 minutes. 5 µl of sample was injected and detective wavelength was 205 nm (stevioside) and 210 nm (rebaudioside A). Peaks were assigned by spiking the samples with standard compounds, comparison of UV spectra and the retention times. The concentration of stevioside in the sample was calculated based on the following formula from JECFA (2008):

\[
C_{stv} (\%) = \frac{W_{stv-std} \times f_{x} \times A_{stv-smp} \times 100}{W_{smp} \times A_{stv-std}}
\]

where \(C_{stv} (\%)\) is the concentration of stevioside, \(W_{stv-std}\) is the weight of the stevioside standard (dried basis), \(W_{smp}\) is the weight of sample (dried basis), \(A_{stv-smp}\) is the peak area of the stevioside in the sample solution, \(A_{stv-std}\) is the peak area of stevioside in the standard solution and \(f_{x}\) is the coefficient value for stevioside that represent the value of 1.
The concentration of rebaudioside A in the sample was calculated by the following formula:

$$C_{\text{reA}} (\%) = \frac{W_{\text{reA-std}} \times A_{\text{x-smp}}}{W_{\text{x-smp}} \times A_{\text{reA-std}}} \times 100$$

where $C_{\text{x}} (\%)$ is the concentration of rebaudioside A, $W_{\text{reA-std}}$ is the weight of the rebaudioside A standard (dried basis), $W_{\text{x-smp}}$ is the weight of sample (dried basis), $A_{\text{x-smp}}$ is the peak area of the rebaudioside A in the sample solution, $A_{\text{reA-std}}$ is the peak area of rebaudioside A in the standard solution.

**ISSR Analysis**

The young leaves of stevia were used to extract genomic DNA through cetyl-trimethyl ammonium bromide (CTAB) method following Lin et al. (2014). Thirty two arbitrary primers (1st Base Laboratories) (25 from Rashid et al., 2013; 6 from Heikal, Badawy, & Hafez, 2008; and 1 from Lata, Chandra, Techen, Wang, & Khan, 2013) were used to assay polymorphism between stevia accessions (Table 2).

**Table 2.** ISSR primers, their sequence and products generated by inter simple sequence repeats (ISSR) in 17 stevia accessions

| ISSR Primer | 5'-3' Nucleotide Sequence | Total number of bands | Total number of polymorphic bands | Polymorphism (%) | Banding range (kb) | Reference |
|-------------|---------------------------|-----------------------|-----------------------------------|------------------|-------------------|-----------|
| IS12        | (AG)8 T                   | 8                     | 7                                 | 88               | 500-1600          | Rashid et al., 2013 |
| IS19        | (CT)8 T                   | 9                     | 7                                 | 78               | 200-2500          | Rashid et al., 2013 |
| IS20        | (CA)8 A                   | 8                     | 7                                 | 88               | 200-1400          | Rashid et al., 2013 |
| IS21        | (CA)8 G                   | 6                     | 5                                 | 83               | 450-1600          | Rashid et al., 2013 |
| IS23        | (GT)8 C                   | 10                    | 9                                 | 90               | 400-2000          | Rashid et al., 2013 |
| IS25        | (TC)8 A                   | 10                    | 10                                | 100              | 300-2500          | Rashid et al., 2013 |
| IS30        | (AC)8 C                   | 9                     | 8                                 | 89               | 300-1400          | Rashid et al., 2013 |
| IS34/1      | (GA)8 CT                  | 13                    | 12                                | 92               | 200-2000          | Rashid et al., 2013 |
| IS34/2      | (GA)6 TTT                 | 8                     | 2                                 | 25               | 500-1500          | Rashid et al., 2013 |
| IS42/2      | (AC)8 TGG                 | 12                    | 10                                | 83               | 300-2200          | Rashid et al., 2013 |
| IS44/1      | (AC)8 CT                  | 6                     | 5                                 | 83               | 700-1800          | Rashid et al., 2013 |
| IS44/2      | (AC)8 TTT                 | 7                     | 6                                 | 86               | 300-1300          | Rashid et al., 2013 |
| IS50        | (GAA)6                    | 17                    | 17                                | 100              | 200-5000          | Rashid et al., 2013 |
| IS52/1      | (TCC)5AC                  | 12                    | 12                                | 100              | 200-5000          | Rashid et al., 2013 |
| IS52/2      | (TCC)5GT                  | 11                    | 8                                 | 73               | 400-2000          | Rashid et al., 2013 |
| IS54        | (AG)5C                    | 12                    | 2                                 | 17               | 400-2500          | Rashid et al., 2013 |
| IS55        | (AG)8A                    | 18                    | 17                                | 94               | 200-1600          | Rashid et al., 2013 |
| IS56        | (TC)8C                    | 9                     | 7                                 | 78               | 300-1600          | Rashid et al., 2013 |
| IS57        | (GA)8CT                   | 12                    | 12                                | 100              | 400-2100          | Rashid et al., 2013 |
| IS70        | (GAA)6                    | 9                     | 9                                 | 100              | 150-3000          | Rashid et al., 2013 |
| IS78        | (AGA)7                    | 15                    | 14                                | 93               | 250-3000          | Rashid et al., 2013 |
| IS83        | (AG)5TT                   | 8                     | 5                                 | 63               | 350-1400          | Rashid et al., 2013 |
| IS85        | (CT)5CAC                  | 12                    | 9                                 | 75               | 350-2500          | Rashid et al., 2013 |
| IS90        | (AG)8G                    | 9                     | 7                                 | 78               | 300-2300          | Rashid et al., 2013 |
| IS94        | (ATG)6                    | 8                     | 4                                 | 50               | 300-900           | Rashid et al., 2013 |
| S4          | (CA)6AC                   | 11                    | 8                                 | 73               | 300-1400          | Heikal, Badawy, & Hafez, 2008 |
| S5          | (CA)6GT                   | 11                    | 7                                 | 64               | 350-1500          | Heikal, Badawy, & Hafez, 2008 |
| S10         | (GA)6CC                   | 9                     | 6                                 | 67               | 300-1300          | Heikal, Badawy, & Hafez, 2008 |
| S11         | (GT)6CC                   | 11                    | 9                                 | 82               | 300-2000          | Heikal, Badawy, & Hafez, 2008 |
| S12         | (CACA)3GC                 | 11                    | 5                                 | 45               | 300-1450          | Heikal, Badawy, & Hafez, 2008 |
| S15         | (GTC)3GC                  | 8                     | 6                                 | 75               | 400-1500          | Heikal, Badawy, & Hafez, 2008 |
| UBC836      | (AG)8CA                   | 13                    | 12                                | 92               | 400-2500          | Lata, Chandra, Techen, Wang, & Khan, 2013 |

Total 332 264 78
**Data Scoring and Analysis**

The frequency of ISSR polymorphism between 17 stevia samples was calculated on the basis of the presence of band as (1) or absence of band as (0) (Guichoux et al., 2011). Only clear reproducible DNA fragments were scored. For similarity purposes, all the fragments were taken into account to ensure the precise estimation of distance (Tucak et al., 2008). Genetic distances were calculated based on Jaccard’s coefficient (Singh & Singh, 2015). Construction of dendrogram was made through un-weighted pair group method of arithmetic averages (UPGMA) by employing the sequential agglomerative hierarchical nested clustering (SAHN) algorithm (Ning et al., 2014) which will group individuals into discrete clusters. NTSYS pc (Numerical Taxonomy System) version 2.1 (Deka, Dadlani, & Sharma, 2016) software package was used for computational calculations.

**RESULTS AND DISCUSSION**

**Morphological Characteristics**

Qualitative morphological characteristics of all 17 stevia accessions are presented in Table 3 and Fig. 1. In terms of plant type, all mother plant accessions were characterised as compact except for Bertam and Eirete II that were showing loose growth. For leaf shape, 9 out of 17 mother plant accessions were obovate except for Nilai and MNQ (ovate), Mergong, Morita III and Native (lanceolate), Bertam and Eirete II (elliptic) and Rasa Sayang (orbicular). For leaf tip, 7 out 17 mother plant accessions exhibited acuminate nature except for MS007, MS012, Nilai and Taman Pertanian (acute), Souq Bukhori, MNQ, Exotic and Kelantan (obtuse), Bangi (retuse) and lastly Rasa Sayang (mucronate). The dominant leaf bases observed among mother plant accessions were attenuate and oblique except for MS007, Bertam, Souq Bukhori and Taman Pertanian (cuneate) and Rasa Sayang (rounded). Lobate crenate and crenate were the dominant leaf margin observed while for Nilai, Langat and Native the leaf margin was characterised as incised, Eirete II and Morita III as serrated and lastly dentate margin for Bertam. Results from qualitative evaluations indicated a diverse array of variability in terms of the leaves characteristics. Tateo et al. (1998) studied 13 types of stevia in a collection in Paraguay and observed the leaf shapes ranged from oblanceolate to obovate to elliptic while leaf margin varied from crenate to dentate to serrated. In another study by Raina, Bhandari, Chand, & Sharma (2013) evaluated 2 different stevia morphotypes known as morphotype A and morphotype B and found that morphotype A was characterized as compact plant feature with leaf shape from obovate to ovate and obtuse tip. Whilst morphotype B was characterized as a loose habit and having obovate leaf shape with an acute tip.

**Table 3. Qualitative morphological characteristics of 17 stevia mother plant accessions**

| Accessions   | Plant type | Shape | Tip   | Base   | Margin       |
|--------------|------------|-------|-------|--------|--------------|
| MS007        | Compact    | Obovate | Acute | Cuneate | Lobate crenate |
| MS012        | Compact    | Obovate | Acute | Oblique | Locate crenate |
| Bangi        | Compact    | Obovate | Retuse | Oblique | Locate crenate |
| Rawang       | Compact    | Obovate | Acuminante | Oblique | Locate crenate |
| Nilai        | Compact    | Ovate  | Acute | Attentuate | Incised   |
| Bertam       | Loose      | Elliptic | Acuminante | Cuneate | Dentate     |
| Souq Bukhori | Compact    | Obovate | Obtuse | Cuneate | Crenate     |
| Taman Pertanian | Compact   | Obovate | Acute | Cuneate | Locate crenate |
| Langat       | Compact    | Obovate | Acuminante | Attentuate | Incised   |
| Mergong      | Compact    | Lanceolate | Acuminante | Oblique | Crenate     |
| R.Sayang     | Compact    | Orbicular | Bucronate | Rounded | Crenate     |
| MNQ          | Compact    | Ovate  | Obtuse | Oblique | Locate crenate |
| Exotic       | Compact    | Obovate | Obtuse | Attentuate | Crenate |
| Kelantan     | Compact    | Obovate | Obtuse | Attentuate | Locate crenate |
| Eirete II    | Loose      | Elliptic | Acuminante | Attentuate | Serrated   |
| Morita III   | Compact    | Lanceolate | Acuminante | Attentuate | Serrated   |
| Native       | Compact    | Lanceolate | Acuminante | Attentuate | Incised   |
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Fig. 1. Qualitative morphological variations of 17 stevia accessions
Quantitative morphological parameters results showed significant differences in all of the observed parameters (Table 4). Data collected 2 months after pruning showed that plant height for all mother plant accessions ranged from 23.4 cm (Morita III) to 36.6 cm (MS012) (Table 4). Apart from MS012 being the tallest, MS007 (31 cm), Souq Bukhori (32.3 cm) and Mergong (31.9 cm) were also among the top performers in terms of plant height. Days of flowering was measured from the time of pruning was conducted on the plants, Eirete II was the earliest flowering accession (35 days) while MS012 (48 days) and Langat (49 days) were the late-flowering accessions. Most accessions took more than 40 days to flowering except Eirete II, Morita III and Native which can be
characterized as the early-flowering accessions. It is highly predictable that under short-day tropical Malaysian environment, stevia exhibit premature or early flowering habit. Typically, under the tropical Malaysian climate, stevia would require about 40-42 days to start flowering. Concomitant with this, unfavourable phenomenon is the fact that the contents of stevioside and rebaudioside A are considerably affected (Brandle & Telmer, 2007). Another disadvantage since stevia is highly priced based on their leaves. The number of stems ranged from 4 stems (Kelantan) to 13 stems (Bertam). Another valuable trait is the leaf yield (leaves number and leaf size) simply because glycosides are most valuable trait in most plant species, the number of stems and leaves, this will indeed bring disadvantages since stevia is highly priced based on their leaves. The number of stems ranged from 4 stems (Kelantan) to 13 stems (Bertam). Another valuable trait is the leaf yield (leaves number and leaf size) simply because glycosides are most abundant in the leaves. Higher leaf yield correlates to higher glycosides content. In terms of the number of leaves, MS012 again was the top performer with 220 leaves followed by MS007 (272 leaves). The poor performer for a number of leaves was Eirete II with merely 90 leaves. Native was also among the lower producing leaves accession with 102 leaves. Previous studies on stevia reported that stevita leaf yield recorded positive correlations with branch number as well as leaf number (Yadav, Singh, Dhyani, & Ahuja, 2011). Another reported that plant height had no significant correlation with production, leaf number or branch number (Yadav, Singh, Dhyani, & Ahuja, 2011). For leaf size character, MS007 had the biggest size recorded at 14.2 cm² and the smallest leaf was recorded by Native with 6.2 cm². MS012 also has fairly big size leaves (13.2 cm²). Eirete II and Morita III (6.1 cm²) were among the smaller sized accessions compared from the rest of the accessions. The biggest stem girth was recorded by MS012 with 2.2 cm. Bangi (1.9 cm) and Taman Pertanian (2 cm) also had fairly equal value recorded for stem girth. Morita III recorded the smallest stem girth at merely 1 cm. Eirete II and Native were also characterized in the smaller stem girth size accessions with stem girth of 1.1 cm. It was obvious that the introduced Paraguayan accessions (Eirete II, Morita III, and Native) performed poorly in regards to overall plant productivity especially in leaf yield.

Table 4. Quantitative morphological characteristics of 17 stevia mother plant accessions

| Accession | Plant Height (cm) | Days to Flowering (days) | Number of Stems | Number of Leaves | Leaf Size (cm²) | Stem Girth (cm) |
|-----------|-------------------|--------------------------|-----------------|-----------------|----------------|-----------------|
| MS007     | 31.0 ± 2.7        | 43.6 ± 2.0               | 5.2 ± 1.2       | 272.6 ± 89.9    | 14.2 ± 1.2     | 1.8 ± 0.5       |
| MS012     | 36.6 ± 4.8        | 48.5 ± 2.5               | 7.0 ± 1.5       | 220.5 ± 76.8    | 13.2 ± 0.4     | 2.2 ± 0.4       |
| Bangi     | 25.2 ± 4.9        | 43.9 ± 2.4               | 8.8 ± 2.9       | 186.5 ± 7.0     | 9.7 ± 4.3      | 1.9 ± 0.5       |
| Rawang    | 31.6 ± 4.1        | 47.4 ± 2.0               | 5.9 ± 2.1       | 146.4 ± 22.5    | 10.4 ± 6.8     | 1.8 ± 0.4       |
| Nilaï     | 32.2 ± 5.4        | 45.0 ± 1.6               | 6.9 ± 1.8       | 255.5 ± 55.3    | 11.5 ± 3.6     | 2.0 ± 0.4       |
| Bertam    | 31.8 ± 7.0        | 47.9 ± 2.3               | 13.7 ± 4.1      | 206.5 ± 63.5    | 5.9 ± 2.4      | 1.5 ± 0.2       |
| Souq Bukhori | 32.3 ± 6.0     | 45.1 ± 2.6               | 7.8 ± 2.1       | 157.5 ± 38.8    | 11.1 ± 2.7     | 1.8 ± 0.4       |
| Taman Pertanian | 29.2 ± 4.6 | 47.5 ± 2.9               | 6.6 ± 2.9       | 178.9 ± 62.2    | 10.7 ± 2.3     | 2.0 ± 0.3       |
| Langat    | 28.4 ± 3.6        | 49.2 ± 2.2               | 6.3 ± 1.6       | 165.0 ± 31.8    | 8.4 ± 5.3      | 1.8 ± 0.4       |
| Mergong   | 31.9 ± 6.5        | 45.5 ± 1.6               | 6.7 ± 2.5       | 168.4 ± 34.2    | 13.3 ± 7.2     | 1.9 ± 0.3       |
| Eirete II | 27.0 ± 3.4        | 35.4 ± 2.3               | 6.6 ± 3.0       | 90.6 ± 15.5     | 6.1 ± 1.8      | 1.1 ± 0.4       |
| Morita III| 23.4 ± 2.9        | 38.7 ± 2.1               | 6.2 ± 2.3       | 122.0 ± 34.7    | 6.1 ± 2.8      | 1.0 ± 0.2       |
| Native    | 24.1 ± 2.8        | 37.3 ± 2.0               | 6.3 ± 1.9       | 102.9 ± 22.8    | 6.2 ± 1.2      | 1.1 ± 0.4       |
| Rasa Sayang | 24.3 ± 3.2       | 41.4 ± 3.0               | 5.0 ± 1.5       | 121.7 ± 32.1    | 9.6 ± 3.4      | 1.2 ± 0.3       |
| MNQ       | 28.0 ± 3.4        | 41.7 ± 2.9               | 5.9 ± 2.2       | 129.4 ± 31.6    | 12.5 ± 4.8     | 1.5 ± 0.2       |
| Exotic    | 29.2 ± 4.9        | 41.4 ± 3.2               | 5.6 ± 1.3       | 150.3 ± 20.8    | 11.7 ± 1.5     | 1.5 ± 0.1       |
| Kelantan  | 30.6 ± 3.2        | 40.0 ± 2.3               | 4.5 ± 1.5       | 135.2 ± 27.3    | 9.0 ± 2.7      | 1.3 ± 0.2       |

Remarks: Different alphabet denotes significant differences at p ≤ 0.05
This study revealed that there were plenty of useful genetic information gained from the assessment conducted. Genetic variation observed was not influenced by any environmental effects such as the type of soil, rainfall or other environmental factors. The stem cutting propagation method practiced in preparing the plants reduced any possible variations that may arise genetically. Through the whole duration of this experiment, all environmental effects were also maintained. There are numerous factors that could affect the growth and flowering of stevia such as day length, soil moisture, radiation, temperature and wind (Yadav, Singh, Dhyani, & Ahuja, 2011). Agronomic yield also mainly depends on the genetic characters of the plant; however phenotypic expression is ultimately governed by climatic and environmental factors (Yadav, Singh, Dhyani, & Ahuja, 2011). This is evident in a research conducted in Egypt which revealed that climate factors such as temperature, day length, and intensity of photoperiod greatly affected stevia production and quality based from the remarkable increase in yield during the summer harvest than that during winter harvest (Yadav, Singh, Dhyani, & Ahuja, 2011).

**Steviol Glycosides Content**

Results from HPLC analysis recorded retention time of 1.52 and 4.01 minutes for stevioside and rebaudioside A respectively. Both calibration curves standards gave good regression value of 0.99962 for stevioside and 0.99958 for rebaudioside A. All the results were statistically significant in comparison to a minimum and maximum values (Table 5). Stevioside content varied from a minimum of 4.54 % (Taman Pertanian) to 20.36 % (Bangi) (Table 5). A study by Raina, Bhandari, Chand, & Sharma (2013) in India reported stevioside content ranging from 4.25-7.32 % and rebaudioside A ranging from 2.01-4.13 %. Stevioside content obtained from this study was higher than those reported by Parris, Shock, & Qian (2016) that ranged from 2.8-5.49 %, Pereira, Storck, Lopes, Martin, & Bisognin (2016) ranging from 6.98-12.16 % and Vouillamoz, Wolfram-Schilling, Carron, & Baroffio (2016) that ranged from 0.3-7.9 %. For rebaudioside A content, MNQ had the highest amount of total (stevioside and rebaudioside A) with 23.79 % while Taman Pertanian contained the lowest amount with merely 5.36 %. The plant compositions with substantially more stevioside than rebaudioside A as observed in this study are similar to other trials reported by Behera et al. (2013), Moraes, Donega, Cantrell, Mello, & McChesney (2013), Pal et al. (2015), Serfaty et al. (2013), and Vasilakoglou, Kalfountzos, Gougoulias, & Reppas (2016). Maximum rebaudioside A: stevioside ratio of 0.33 was observed in MNQ while the minimum ratio of 0.02 was observed in Nilai and Eirete II.

The variation in steviol glycosides content in stevia mother plant collection was linked to the open-pollination nature stevia (Tateo et al., 1998). Rebaudioside A is more preferred than stevioside due to its most desirable flavor profile (Yadav, Singh, Dhyani, & Ahuja, 2011). Stevioside is reported to be found most abundant (make up to 60-70 % of the total glycosides content) but it is less preferred because of the bitter aftertaste (Tavarini & Angelini, 2013). For rebaudioside A, it is found in 30-40 % from the total glycosides and being the sweetest (Yadav, Singh, Dhyani, & Ahuja, 2011). The ratio between rebaudioside A and stevioside is the accepted measurement to assess the sweet quality in stevia. The bitter aftertaste of stevioside can be masked by the sweetness of rebaudioside A if the ratio is equal or superior to 1 (Yadav, Singh, Dhyani, & Ahuja, 2011). Previous studies also indicated that stevioside and rebaudioside A are negatively correlated (Yadav, Singh, Dhyani, & Ahuja, 2011). Results from a crossbreeding stevia population consisting of different profiles of steviol glycosides suggested that rebaudioside A presence is highly influenced by a single dominating gene while its content are controlled by a higher numbers of loci (Brandle, 1999). Therefore, stevia has high breeding potential and subjected to multitude of breeding improvement through selection towards the release of improved varieties (Gupta, Purwar, Sundaram, & Rai, 2013).

**ISSR Analysis**

Results from the present study, revealed that ISSR could clearly distinguish the stevia accessions with high polymorphism level. There were 332 clear and reproducible bands produced using 32 ISSR primers in 17 stevia accessions (Table 2) and the ISSR profile and polymorphism produced are
shown in (Fig. 2). Number of total amplified bands ranged from 6 (IS21and IS44/1) to 18 (IS55) with an average of 10 bands per primer (Table 2). The amplified products varied in sizes ranging from 150 and 5000 bp.

From 332 amplified bands, 264 were identified as polymorphic bands. The number of polymorphic fragments ranged from 2 (IS34/2 and IS54) to 17 (IS50 and IS55) bands. The average number of polymorphic fragments per primer was 8.25. Relative polymorphism was lowest for primer IS54 (17 %) and highest for primer IS25, IS50, IS52/1, IS57 and IS70 (100 %). The percentage of polymorphism across all the samples was 78 %. Conventionally stevia identification is based on morphological characters (Abdullateef & Osman, 2011; Anami et al., 2010). The limitation with morphological characterization is that it is restricted to a particular reproductive stage and is highly affected by the environment, thus molecular identification and characterization are more reliable. The use of ISSR in the identification of genetic relationship among stevia was also studied by Heikal, Badawy, & Hafez (2008) and Sharma, Kaur, & Era (2016). This result suggested profound genetic diversity in stevia accessions. This great genetic polymorphism may also be accounted to the high degree of heterozygosity because of the out-crossing nature of stevia.

### Table 5. Steviol glycosides of 17 stevia mother plant accessions

| Accession | Stevioside (%) | Rebaudioside A (%) | Total (stevioside + rebaudioside A) (%) | Rebaudioside A / Stevioside |
|-----------|----------------|--------------------|---------------------------------------|-----------------------------|
| MS007     | 5.35± ± 0.79   | 0.34± ± 0.04       | 5.69± ± 0.83                          | 0.06± ± 0.00                |
| MS012     | 11.80± ± 4.39  | 0.75± ± 0.00       | 12.55± ± 4.39                         | 0.07± ± 0.03                |
| Bangi     | 20.36± ± 3.39  | 3.43± ± 0.37       | 23.79± ± 3.76                         | 0.17± ± 0.01                |
| Rawang    | 9.00± ± 3.02   | 0.31± ± 0.02       | 9.31± ± 3.01                          | 0.04± ± 0.01                |
| Nilia     | 18.62± ± 3.84  | 0.30± ± 0.01       | 18.92± ± 3.83                         | 0.02± ± 0.00                |
| Bertam    | 5.48± ± 0.24   | 1.24± ± 0.09       | 6.72± ± 0.33                          | 0.23± ± 0.01                |
| Souq Bukhori | 9.78± ± 3.12 | 0.38± ± 0.02       | 10.16± ± 3.15                         | 0.04± ± 0.01                |
| Taman Pertanian | 4.54± ± 0.37 | 0.82± ± 0.01       | 5.36± ± 0.38                          | 0.18± ± 0.01                |
| Langat    | 6.15± ± 1.81   | 0.53± ± 0.06       | 6.68± ± 1.88                          | 0.09± ± 0.02                |
| Mergong   | 12.41± ± 1.71  | 0.75± ± 0.15       | 13.16± ± 1.86                         | 0.06± ± 0.00                |
| Eirete II | 15.9± ± 2.16   | 0.32± ± 0.01       | 16.29± ± 2.17                         | 0.02± ± 0.00                |
| Morita III| 11.78± ± 1.88  | 1.38± ± 0.03       | 13.16± ± 1.85                         | 0.12± ± 0.02                |
| Native    | 15.42± ± 2.04  | 1.65± ± 0.46       | 18.09± ± 1.48                         | 0.09± ± 0.03                |
| Rasa Sayang | 13.61± ± 4.52 | 0.59± ± 0.04       | 14.20± ± 4.56                         | 0.05± ± 0.01                |
| MNQ       | 6.91± ± 1.35   | 2.04± ± 0.84       | 8.95± ± 0.51                          | 0.33± ± 0.19                |
| Exotic    | 8.77± ± 0.35   | 0.71± ± 0.07       | 9.48± ± 0.42                          | 0.08± ± 0.00                |
| Kelantan  | 7.00± ± 1.43   | 0.70± ± 0.12       | 7.69± ± 1.31                          | 0.11± ± 0.04                |

Remarks: Different alphabet denotes significant differences at $p \leq 0.05$
Fig. 2. ISSR pattern of stevia accessions generated from IS25, IS50, IS52/1, IS57 and IS70
Phylogenetic tree analysis showed the grouping of all stevia accessions into 3 major groups (1-3) at the similarity coefficient of 0.44 (Fig. 3). It is obvious that Bertam was not included in any of the main groups and appeared as a separate branch at similarity coefficient of 0.44 as the most genetically distant of all in group III. Group I consisted of the rest of the remaining 16 stevia accessions and further separated into 5 subgroups (1-5) at similarity coefficient of 0.48. Souq Bukhori was placed into group II at similarity coefficient of 0.46. The first subgroup at the coefficient value of 0.60 included 2 sub-clusters. Rawang and Nilai, very close to each other with Bangi, MS007, and MS012 with Langat joining at the similarity coefficient of 0.67 were presented as one sub-cluster. Another sub-cluster consisted of only Taman Pertanian. Subgroup 2 was further divided into 2 sub-clusters at coefficient value of 0.58. Native and Kelantan were grouped in the first sub-cluster with similarity coefficient of 0.60. Another sub-cluster comprised of only Exotic. The third subgroup was further separated into 2 sub-clusters at the similarity value of 0.59. Mergong and Eirete II were placed in the first sub-cluster at similarity coefficient of 0.63.Morita III was solely included in the second sub-cluster. Rasa Sayang was comprised in subgroup 4 at similarity value of 0.53. MNQ was placed in subgroup 5 with the similarity coefficient of 0.47. Bertam separated from the rest of the groups as a distinct accession. From the rest of stevia accessions, only Bertam was acquired from a highland area. This suggests that the variation detected at genetic level may be caused by the different place of the collection with different geographical variations. The genetic diversity of a species is affected by a number of evolutionary factors including mating systems, seed dispersal, gene flow, natural selection, geographical range and the diversity center (Wu, Shen, Zhang, Wang, & Sun, 2014). Bertam could also be derived from crosses that involve different stevia which are not similar to any of our stevia mother plants collection. Results obtained from phylogenetic analysis can help to devise proper breeding strategies, especially in the hybridization programme to produce promising stevia hybrids. Stevia accessions listed in subgroup 1 (MS007, MS012, Bangi, Rawang, Nilai, Langat and Taman Pertanian) are among the best-proposed accessions to be crossed with Bertam to produce stevia hybrids based on their coefficient similarity indices.
Fig. 3. Dendrogram illustrating genetic relationship among 17 stevia accessions generated from
CONCLUSION AND SUGGESTION

It is feasible to conclude that the stevia accessions studied have sufficient genetic variability which is useful and valuable for any breeding programme in the near future towards the development of improved varieties of specific characteristics. The results from our evaluation show that there is a substantial variability in the stevia accessions collection with regards to morphological, chemical and genetics. Hence, the possibility for further improvement using these variations is wide. Therefore, economical traits of stevia such as higher leaf yield which is important towards the development of stevia-based industry which relies heavily on leaf production can possibly be improved for their quality and quantity through selection. As a conclusion, a number of promising accessions such as MS007, MS012, Bangi and Bertam can be further utilized in a systematic stevia breeding programme with good introgression from Paraguayan varieties to get improved overall traits in the future selection made.

REFERENCES

Abdullateef, R. A., & Osman, M. (2011). Influence of genetic variation on morphological diversity in accessions of Stevia rebaudiana Bertoni. International Journal of Biology, 3(3), 66–72. http://doi.org/10.5539/ijb.v3n3p66

Anami, E.T., Poletine, J.P., Gonçalves-Vidigal, M.C., Vidigal Filho, P.S., Lacanallo, G.F., Kvitshalc, M.V., & Gonela, A. (2010). Characterization and genetic divergence in Stevia rebaudiana (Bert.) Bertoni clones based in agronomical and morphological characteristics. Journal of Food, Agriculture and Environment, 8(3-4 part 1), 463–469. Retrieved from https://www.researchgate.net/publication/286389928_Characterization_and_genetic_divergence_in_Stevia_rebaudiana_Bert_Bertoni_clones_based_in_agronomical_and_morphological_characteristics

Bajpai, P. K., Warghat, A. R., Sharma, R. K., Yadav, A., Thakur, A. K., Srivastava, R. B., & Stobdan, T. (2014). Structure and genetic diversity of natural populations of Morus alba in the trans-Himalayan Ladakh region. Biochemical Genetics, 52(3–4), 137–152. http://doi.org/10.1007/s10528-013-9634-5

Balcázar-Vargas, M. P., Peñuela-Mora, M. C., van Andel, T. R., & Zuidema, P. A. (2012). The quest for a suitable host: Size distributions of host trees and secondary hemiepiphytes search strategy. Biotropica, 44(1), 19–26. http://doi.org/10.1111/j.1744-7429.2011.00767.x

Barthe, S., Gugerli, F., Barkley, N. A., Maggia, L., Cardi, C., & Scotti, I. (2012). Always look on both sides: Phylogenetic information conveyed by simple sequence repeat allele sequences. PLoS ONE, 7(7), e40699. http://doi.org/10.1371/journal.pone.0040699

Behera, M. S., Verma, O. P., Mahapatra, P. K., Singandhupe, R. B., Kumar, A., Kannan, K., & Brahmanand, P. S. (2013). Effect of fertigation on stevia (Stevia rebaudiana) under drip irrigation. Indian Journal of Agronomy, 58(2), 243–250. https://doi.org/10.5958/j.4.3.015

Brandle, J. (1999). Genetic control of rebaudioside A and C concentration in leaves of the sweet herb, Stevia rebaudiana. Canadian Journal of Plant Science, 79(1), 85–91. https://doi.org/10.4141/P98-048

Brandle, J. E., & Telmer, P. G. (2007). Stevioside biosynthesis. Phytochemistry, 68(14), 1855–1863. http://doi.org/10.1016/j.phytochem.2007.02.010

Ceunen, S., Werbrouck, S., & Geuns, J. M. C. (2012). Stimulation of steviol glycoside accumulation in Stevia rebaudiana by red LED light. Journal of Plant Physiology, 169(7), 749–752. http://doi.org/10.1016/j.jplph.2012.01.006

Chotikadachanarong, K., & Dheeranupattana, S. (2013). Micropropagation and acclimatization of Stevia rebaudiana Bertoni. Pakistan Journal of Biological Sciences, 16(17), 887–890. http://doi.org/10.3923/pjbs.2013.887.890

Deka, S. D., Dadlani, M., & Sharma, R. (2016). Diversity study in capsicum using numerical taxonomy. Sabrao Journal of Breeding and Genetics, 48(3), 277–284. Retrieved from http://www.sabrao.org/journals/Jul-Sept-2016/SABRAO-J-Breed-Genet-48-3-277-284-Deka.pdf

Goettemoeller, J., & Ching, A. (1999). Seed germination in Stevia rebaudiana. In J. Janick (Ed.), Perspectives on new crops and new uses (pp. 510–511). Alexandria, VA: ASHS Press.
Halimaton Saadiah Othman et al.: Morphological, Chemical and Molecular Variabilities of Stevia rebaudiana Bertonie...

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Léger, P., Lepais, O., ... Petit, R. J. (2011). Current trends in microsatellite genotyping. *Molecular Ecology Resources, 11*(4), 591-611. http://doi.org/10.1111/j.1755-0998.2011.03014.x

Gupta, E., Purwar, S., Sundaram, S., & Rai, G. K. (2013). Nutritional and therapeutic values of *Stevia rebaudiana*: A review. *Journal of Medicinal Plants Research, 7*(46), 3343-3353. https://doi.org/10.5897/JMPR2013.5276

Gupta, S., Srivastava, M., Mishra, G. P., Naik, P. K., Chauhan, R. S., Tiwari, S. K., ... Singh, R. (2008). Analogy of ISSR and RAPD markers for comparative analysis of genetic diversity among different *Jatropha curcas* genotypes. *African Journal of Biotechnology, 7*(23), 4230-4243. Retrieved from https://www.ajol.info/index.php/ajb/article/download/59558/47849

Heikal, A. H., Badawy, O. M., & Hafez, A. M. (2008). Genetic relationships among some Stevia (*Stevia rebaudiana* Bertoni) accessions based on ISSR analysis. *Research Journal of Cell and Molecular Biology, 2*(1), 1-5. Retrieved from https://scholar.google.com/scholar?cluster=16600501505645326247&hl=en&oi=scholarr

JECFA. (2008). Steviol glycosides. FAO JECFA Monographs 5. Retrieved from http://www.fao.org/ag/agn/jecfa-additives/specs/monograph5/additive-442-m5.pdf

Lata, H., Chandra, S., Techen, N., Wang, Y.-H., & Khan, I. A. (2013). Molecular analysis of genetic fidelity in micropropagated plants of *Stevia rebaudiana* Bert. using ISSR marker. *American Journal of Plant Sciences, 4*(May), 964–971. http://doi.org/10.4236/ajps.2013.45119

Lin, T., Zhu, G., Zhang, J., Xu, X., Yu, Q., Zheng, Z., ... Huang, S. (2014). Genomic analyses provide insights into the history of tomato breeding. *Nature Genetics, 46*(11), 1220–1226. http://doi.org/10.1038/ng.3117

Madan, S., Ahmad, S., Singh, G. N., Kohli, K., Kumar, Y., Singh, R., & Garg, M. (2010). *Stevia rebaudiana* (Bert.) Bertoni - A review. *Indian Journal of Natural Products and Resources, 1*(3), 267–286. http://doi.org/10.1016/S0031-9422(03)00426-6

Martins-Lopes, P., Lima-Brito, J., Gomes, S., Meirinhos, J., Santos, L., & Guedes-Pinto, H. (2007). RAPD and ISSR molecular markers in *Olea europaea* L.: Genetic variability and molecular cultivar identification. *Genetic Resources and Crop Evolution, 54*(1), 117–128. https://doi.org/10.1007/s10722-005-2640-7

Mehta, B. K., Jain, D. C., Misra, H., Soni, M., Silawat, N., & Mehta, D. (2011). Antidiabetic activity of medium-polar extract from the leaves of *Stevia rebaudiana* Bert. (Bertoni) on alloxan-induced diabetic rats. *Journal of Pharmacy and Bioallied Sciences, 3*(2), 242. http://doi.org/10.4103/0975-7406.80779

Mogra, R., & Dashora, V. (2009). Exploring the use of *Stevia rebaudiana* as a sweetener in comparison with other sweeteners. *Journal of Human Ecology, 25*(2), 117–120. http://doi.org/10.1080/09709274.2009.1190614

Moraes, R. M., Donega, M. A., Cantrell, C. L., Mello, S. C., & McChesney, J. D. (2013). Effect of harvest timing on leaf production and yield of diterpine glycosides in *Stevia rebaudiana* Bert.: A specialty perennial crop for Mississippi. *Industrial Crops and Products, 51*, 385–389. http://doi.org/10.1016/j.indcrop.2013.09.025

Nazneen, H., Reddy, P. V., & Reddy, S. K. (2016). Molecular analysis of genetic fidelity of *Stevia rebaudiana* Bert. using RAPD markers. *World Journal of Pharmaceutical Research, 5*(4), 750-759. https://doi.org/10.20959/wjpr20164-5771

Ning, H., Wang, W., Zheng, C., Li, Z., Zhu, C., & Zhang, Q. (2014). Genetic diversity analysis of sedges (Carex spp.) in Shandong, China based on inter-simple sequence repeat. *Biochemical Systematics and Ecology, 56*, 158–164. http://doi.org/10.1016/j.bse.2014.05.014

Owens, A., Cieslak, M., Hart, J., Classen-Bockhoff, R., & Prusinkiewicz, P. (2016). Modeling dense inflorescences. *ACM Transactions on Graphics, 35*(4), 1–14. http://doi.org/10.1145/2897824.2925982

Pal, P. K., Mahajan, M., Prasad, R., Pathania, V., Singh, B., & Ahuja, P. S. (2015). Harvesting regimes to optimize yield and quality in annual and perennial *Stevia rebaudiana* under sub-tropical conditions. *Industrial Crops and Products, 65*, 556–564. http://doi.org/10.1016/j.indcrop.2014.09.060

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Halimaton Saadiah Othman et al.: Morphological, Chemical and Molecular Variabilities of Stevia rebaudiana Bertoni......

Parris, C. A., Shock, C. C., & Qian, M. (2016). Dry leaf and steviol glycoside productivity of Stevia rebaudiana in the Western United States. HortScience, 51(10), 1220–1227. http://doi.org/10.21273/HORTSCI11149-16

Pereira, C., Storck, L., Lopes, S. J., Martin, T. N., & Bisognin, D. A. (2016). Dry biomass and glycosides yield from Stevia rebaudiana leaves under different harvesting times. Biosciencia Journal, 32(6), 1462-1471. https://doi.org/10.14393/BJ-v32n6a2016-31574

Prakash, I., Marksosyan, A., & Bunders, C. (2014). Development of next generation stevia sweetener: Rebaudioside M. Foods, 3, 162-175. Retrieved from http://www.mdpi.com/2304-8158/3/1/162

Raina, R., Bhandari, S. K., Chand, R., & Sharma, Y. (2013). Strategies to improve poor seed germination in Stevia rebaudiana, a low calorie sweetener. Journal of Medicinal Plants Research, 7(24), 1793–1799. http://doi.org/10.5897/JMPR12.226

Rajasekaran, T., Girdhar, P., & Ravishankar, G. A. (2007). Production of steviosides in ex vitro and in vitro grown Stevia rebaudiana Bertoni. Journal of the Science of Food and Agriculture, 87(3), 420–424. http://doi.org/10.1002/jsfa.2713

Rashid, R. A., Mohamad, A., Awang, M. R., Mutaat, H. H., Mohamad, S. A., Hasan, A. A., ... Mohd Nahar, S. K. (2013). Isolation and optimization of inter-simple sequence repeat (ISSR) technique for Pleurotus sajor caju towards environmental study in support of the country’s nuclear power programme. Jurnal Sains Nuklear Malaysia, 25(1), 1–8. Retrieved from https://jsnm.nuclearmalaysia.gov.my/index.php/jsnm/article/download/76/77/

SAS Institute Inc. (1999). SAS/STAT® 8.02 User’s Guide. Cary, NC: SAS institute, Inc.

Serfaty, M., Ibdah, M., Fischer, R., Chaimovitsh, D., Saranga, Y., & Dudai, N. (2013). Dynamics of yield components and stevioside production in Stevia rebaudiana grown under different planting times, plant stands and harvest regime. Industrial Crops and Products, 50, 731–736. http://doi.org/10.1016/j.indcrop.2013.08.063

Sharma, N., Kaur, R., & Era, V. (2016). Potential of RAPD and ISSR markers for assessing genetic diversity among Stevia rebaudiana Bertoni accessions. Indian Journal of Biotechnology, 15, 95-100. Retrieved from https://pdfs.semanticscholar.org/1b3a/154c6351c7dc6d2a249fdda51f9766e2b41.pdf

Singh, B. D., & Singh, A. K. (2015). Marker-assisted plant breeding: Principles and practices. New Delhi, IN: Springer. http://doi.org/10.1007/978-81-322-2316-0

Singh, P., & Dwivedi, P. (2014). Two-stage culture procedure using thiadiazuron for efficient micropropagation of Stevia rebaudiana, an anti diabetic medicinal herb. 3 Biotech, 4(4), 431-437. https://doi.org/10.1007/s13205-013-0172-y

Steinmetz, W. E., & Lin, A. (2009). NMR studies of the conformation of the natural sweetener rebaudioside A. Carbohydrate Research, 344(18), 2533–2538. http://doi.org/10.1016/j.carres.2009.10.005

Tan, S. L., Ghawas, M. M., Najib, M. Y. M., & Zawayi, M. (2008). Preliminary evaluation and selection of stevia under Malaysian conditions. Journal of Tropical Agriculture and Food Science, 36(2), 1–7. Retrieved from http://ejtafs.mardi.gov.my/efafs/36-2/Stevia.pdf

Tateo, F., Mariotti, M., Bononi, M., Lubian, E., Martello, S., Cornara, L. (1998). Stevioside content and morphological variability in a population of Stevia rebaudiana (Bertoni) Bertoni from Paraguay. Italian Journal of Food Science, 10, 261-267.

Tavarini, S., & Angelini, L. G. (2013). Stevia rebaudiana Bertoni as a source of bioactive compounds: the effect of harvest time, experimental site and crop age on steviol glycoside content and antioxidant properties. Journal of the Science of Food and Agriculture, 93(9), 2121–2129. https://doi.org/10.1002/jsfa.6016

Thiyagarajan, M., & Venkatachalam, P. (2015). Assessment of genetic and biochemical diversity of Stevia rebaudiana Bertoni by DNA fingerprinting and HPLC analysis. Annals of Phytomedicine, 4(1), 79-85. Retrieved from http://ukaazpublications.com/attached/publications/10-20Article%20(79-85).pdf

Tucak, M., Popović, S., Ćupić, T., Grjišić, S., Bolarić, S., & Kozumplik, V. (2008). Genetic diversity of alfalfa (Medicago spp.) estimated by molecular markers and morphological characters. Periodicum
Halimaton Saadiah Othman et al.: Morphological, Chemical and Molecular Variabilities of Stevia rebaudiana Bertoni......

Biologorum, 110(3), 243–249. Retrieved from https://hrcak.srce.hr/file/51848

Vasilakoglou, I., Kalfountzos, D., Gougoulias, N., & Reppas, C. (2016). Productivity of two stevia varieties under reduced irrigation and fertilization inputs. Archives of Agronomy and Soil Science, 62(4), 457–472. http://doi.org/10.1080/03650340.2015.1060554

Vouillamoz, J. F., Wolfram-Schilling, E., Carron, C.-A., & Baroffio, C. A. (2016, June 19-23). FSL 4: Agronomical and phytochemical evaluation of Stevia rebaudiana genotypes. Paper presented at 6th International Symposium Breeding Research on Medicinal and Aromatic Plants, BREEDMAP 6, Quedlinburg, Germany. https://doi.org/10.5073/jka.2016.453.028

Wu, F. Q., Shen, S. K., Zhang, X. J., Wang, Y. H., & Sun, W. B. (2014). Genetic diversity and population structure of an extremely endangered species: the world’s largest Rhododendron. AoB PLANTS, 7, plu082. http://doi.org/10.1093/aobpla/plu082

Yadav, A. K., Singh, S., Dhyani, D., & Ahuja, P. S. (2011). A review on the improvement of stevia [Stevia rebaudiana (Bertoni)]. Canadian Journal of Plant Science, 91(1), 1–27. http://doi.org/10.4141/cjps10086

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