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Karyomorphology, genome size, and variation of antioxidant in twelve berry species from Iran

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Abstract. Twelve berry species, including *Rubus fruticosus* cv. Qaemshahr, *Rubus occidentalis* cv. Qaemshahr, and *Morus alba* cv. Mashhad, *Morus rubra* cv. Karaj, *Fragaria vesca* subsp. *vesca*, *Ribes nigrum*, *Ribes rubrum*, *Ribes uva-crispa*; *Lycium barbarum*, *Lycium infaustum*, *Lycium ruthenicum*, and *Vaccinium corymbosum* were surveyed for karyomorphology analysis, monoploid genome size, and antioxidant activity. Results indicated that all species were diploid ($2n = 2x = 14, 16, 24, \text{ and } 28$). Among these species, chromosome counts and karyomorphology parameters of three cultivars including: *R. fruticosus* cv. Qaemshahr ($2n = 2x = 14$), *R. occidentalis* cv. Qaemshahr ($2n = 2x = 14$), and *M. alba* cv. Mashhad ($2n = 2x = 28$) are reported here for the first time. The flow cytometric mean monoploid $2Cx$ DNA of berry species was 2.35 pg, varied from 0.68 (*Rubus occidentalis* cv. Qaemshahr) to 5.15 pg (*Lycium ruthenicum*). The average antioxidant capacity of berry species was obtained $32.8 \mu\text{mol g}^{-1}$. Their average total phenol and flavonoid contents were 4.98 mg g^{-1} ($3.08\text{-}8.61 \text{ mg g}^{-1}$), and 5.18 ($2.47\text{-}10.63 \text{ mg g}^{-1}$), respectively.

Keywords: Blueberry, Raspberry, Cytogenetic, Antioxidant, Karyomorphology, Flow cytometry.

INTRODUCTION

Berries are economically important crop in many countries (Umdale *et al.* 2020). Interest in berries has recently increased because they are excellent sources of health-promoting vitamins, anti-oxidants, polyphenols, especially anthocyanins, micronutrients, fiber, and other valuable nutrients (Song and Sing, 2004). Berries are low in calories and are high in moisture and fiber (Basu *et al.* 2010). They contain natural antioxidants such as vitamins C and E, and micronutrients such as folic acid, calcium, selenium, alpha and beta carotene, and lutein (Basu 2019). However, berry production is deficient, even though soil and climate conditions in Iran are excellent for the intensive cultivation of berry fruits (Nestby *et al.* 2019). Some of the berry cultivars that

have recently been introduced to Iran are potentially profitable alternative and non-conventional fruit crops. Among those are cultivars of the blueberry (*Vaccinium corymbosum* L.), the goji berry (*Lycium barbarum*, *L. infaustum*, *L. ruthenicum*), and the raspberry (*Rubus occidentalis*) (Ahmadu and Ahmad 2021). In Iran, the production of blackberry (*Rubus fruticosus*) relies mainly on the collection of the berries from stands of native varieties that grow wild in the mountains. Habitat destruction has led to a reduction in the supply of native blackberries. Although these native wild varieties are not as suitable for intensive culture as the new cultivars, they are still very valuable high dietary value and breeding potential. They can be a versatile raw material for the food processing and pharmaceutical industries. Recently, interest in growing various berry fruits has been increasing among either home gardeners, or small farmers. Berry culture is increasing in the country's mountainous, where soil and climate conditions are more favorable for intensive berry culture than in the lowland areas (Nestby *et al.* 2019).

The consumption of berry fruits and their contribution to improving cardiovascular health is a significant issue (Blumberg *et al.* 2013). The commonly consumed berries in the United States, including blackberry, black raspberry, blueberry, goji berry, cranberry, red raspberry, and strawberries (Yang *et al.* 2019). Display quotations of over 40 words or as needed.

Berry fruits are deciduous shrubbery that grows in different parts of the world (Donno *et al.* 2015). For instance, goji berry grows in China, Tibet, Argentina, Chile, Southern Africa, and other parts of Asia, and its fruits are 1-2 cm long, bright orange-red ellipsoid berries. Goji berry is widely distributed in warm regions of the world, in particular, in the Mediterranean area and Southwest and Central Asia. It is also cultivated in North America and Australia as a hedge plant (Potterat 2010). Mulberry (*Morus alba*) is native to China, but is also found worldwide on not native continents, such as Europe, Africa, North America, and South America. Currant (*Ribes* spp.), is widely cultivated across temperate Europe, Russia, New Zealand, parts of Asia, and to a lesser extent North America (Steffen *et al.* 2015). The main centers of diversity for blueberry are distributed in Europe (Poland, Germany, France, The Netherlands, Spain, and Sweden), New Zealand, Mexico, and North America.

Karyotype analysis and chromosome observation are necessary to elucidate phylogenetic relationships, structure, function, organization, and evolution of plant genes and genomes (Amosova *et al.* 2019). For those reasons, many studies must be performed to observe plant chromosomes. Higher similarity in chromosome shapes

suggests a closer phylogenetic relatedness. The base chromosome number in *Rubus* spp. (*R. fruticosus*, and *R. occidentalis*), *Ribes* spp. (*R. nigrum*, *R. rubrum*, and *R. uva-crispa*), Lyceae (*L. barbarum*, *L. infaustum*, and *L. ruthenicum*), Moraceae (*M. alba*, and *M. rubra*) is $x = 7, 8, 12,$ and $14,$ respectively. The base chromosome number in strawberry (*Fragaria vesca*) and blueberry (*Vaccinium corymbosum*) is $x = 7,$ and $14,$ respectively (Zong *et al.* 2021). Most species are diploid with $2n = 24.$ Cytological information, including the number of chromosome and karyotypes, is available for many American and Asian *Lycium* and several species in Southern African (Bernardello *et al.* 2008).

Monoploid genome size in the form of base-pair was calculated based on the formula proposed by Doležel *et al.* (2003), when 1 pg of DNA represents 978 mega base pairs (Mbp). Monoploid genome size was considered as the amount of DNA of one chromosome set, $1Cx$ -value, with chromosome base number $x,$ and holoploid genome size as the amount of DNA of the whole chromosome complement, $1C$ -value, with chromosome number $n,$ irrespective of the degree of generative polyploidy (Greilhuber *et al.* 2005; Mahdavi and Karimzadeh 2010; Karimzadeh *et al.* 2011; Abedi *et al.* 2015; Tavan *et al.* 2015). In recent decades, significant progress has been made in the use of flow cytometry in various fields of botany. This growth is due to the advantages of flow cytometry such as high speed, ease of sample preparation, and analysis of inactive mitotic tissues. Despite these advantages, the need for fresh plant materials may often prevent the further development of flow cytometry in field research (Suda and Trávníček 2006). Flow cytometry is known as the most powerful, reliable, and quick method for estimating the genome size (2C DNA) for a wide range of plant communities (e.g. Garcia *et al.* 2004; Doležel and Bartoš 2005; Doležel *et al.* 2007; Loureiro *et al.* 2007; Mahdavi and Karimzadeh 2010; Karimzadeh *et al.* 2010, 2011; Abedi *et al.* 2015; Tavan *et al.* 2015; Tarkesh Esfahani *et al.* 2016, 2020; Javadian *et al.* 2017; Hamidi *et al.* 2018; Zarabizadeh *et al.* 2022). The term "C-value" refers to the constant amount of DNA of an unreplicated haploid chromosome complement (Swift 1950). Monoploid genome size was considered as the amount of DNA of one chromosome set, $1Cx$ -value, with chromosome base number x (Greilhuber *et al.* 2005). The mean monoploid $2Cx$ DNA value was 2.35 pg. On the other hand, the monoploid genome size ($1Cx$ DNA) varied from 420.54 Mbp (S3; *Rubus occidentalis* cv. Qaemshahr) to 2518.35 Mbp (S9; *Lycium ruthenicum*). Reports of $2Cx$ DNA in some berry species have been reported, including S3; *Rubus occidentalis* = 0.60 pg, S5; *Ribes rubrum* = 1.94 pg, and S6; *Ribes*

uva-crispa = 1.88 pg (Meng and Finn 2002; Chiche *et al.* 2003).

Antioxidants are compounds that effectively prevent oxidation in a variety of ways and, by slowing down the oxidation rate, significantly increase the oxidation period (Dorman *et al.* 2003). From one perspective, antioxidants fall into two main categories: synthetic and natural. Phenolic compounds are one of the best sources of natural antioxidants (Dorman *et al.* 2003). Because the use of such plant resources is effective in delaying oxidation and reducing chronic diseases, mutations, and cancer (Briskin 2000). In new research works in the field of natural antioxidants, much attention has been paid to essential oils and extracts of medicinal plants because a wide range of medicinal plants and their aromatic compounds as natural sources with antioxidant properties, (Moure *et al.* 2001; Tepe *et al.* 2006). It is generally recognized that oxygen free radicals (ROS) are associated with many potential risks, including Parkinson's disease, cancer, Alzheimer's, as well as aging (Liochev 2013; Kim *et al.* 2016). Berry species are popular around the world as a 'Super fruit' due to their nutritional value, elevated levels of bioactive phenolic molecules, and excellent sensory evaluation (Kalt *et al.* 2020). In addition to these essential nutrients, berries contain a wide range of antioxidant phenolic molecules such as phenolic acids, flavonoids, flavonols, flavanols, anthocyanins, proanthocyanidins, and ellagitannins (Prior *et al.* 1998; Piljac-Zegarac *et al.* 2009; Nile and Park 2014; Skrovankova *et al.* 2015). Cranberries ranked first in polyphenol content among the commonly consumed fruits in North America which relate to high antioxidant activity (Vinson *et al.* 2001). The Consumption of lingonberries and bilberries has been proved in preventing human cancer which

is ascribed to the high levels of phenolic and anthocyanin compounds (Faria *et al.* 2010; Yang and Kortessniemi 2015). The average total antioxidant capacity, total phenol content, total flavonoid content of berries were 32.79, 4.98, and 5.18 $\mu\text{mol g}^{-1}$, respectively. In the study of Islam *et al.* (2017) on genus *Lycium*, the mean of the antioxidant activity for red goji berry (S7; *Lycium barbarum*) and black goji berry (S9; *Lycium ruthenicum*) were measured as 16.65 and 34.28 $\mu\text{mol g}^{-1}$, respectively. In the report of Mustafa Ahmed *et al.* (2022) on genus *Vaccinium*, the mean of total phenol content, total flavonoid content, and antioxidant capacity were reported as 3.73 mg g^{-1} , 2.57 mg g^{-1} , and 17.67 $\mu\text{mol g}^{-1}$, respectively.

In the present study, we provide a detailed karyotype analysis for 12 berries species. Our goals were to confirm the number of chromosomes, karyotype determination, estimate the nuclear genome size, assess the phenolic profile, antioxidant properties, and flavonoid content of these twelve species, and provide scientific insight into the phenolic and antioxidant functions of these species to consumers and nutraceutical industry.

MATERIALS AND METHODS

Plant materials

Seeds were kindly provided by the Cultivation Development Company of Berries (Mazandaran, Iran). The culture medium used was MS (Murashige and Skoog 1962) with macroelements at 1/3 (Rache and Pacheco 2010), 1/8, and 1/16 of its original concentration (NH_4NO_3 : 1650 mg l^{-1} , KNO_3 : 1900 mg l^{-1} , KH_2PO_4 : 170 mg l^{-1} , $\text{CaCl}_2\text{H}_2\text{O}$: 440 mg l^{-1} and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$: 370

Table 1. Specifications of berry species collection sites (Berry spp.) used in this research.

Species codes	English name	Scientific name	Localities	Longitude (E); Latitude (N)	Altitude (m)
S1	Strawberry	<i>Fragaria vesca</i> subsp. <i>vesca</i>	Iran, Mazandaran, sari	52°59'42.83"; 36°32'25.67"	83
S2	Blackberry	<i>Rubus fruticosus</i> cv. Qaemshahr	Iran, Qaemshahr	52°51'28.29"; 36°27'19.11"	282
S3	Black raspberry	<i>Rubus occidentalis</i> cv. Qaemshahr	Iran, Qaemshahr	52°51'28.29"; 36°27'19.11"	282
S4	Black currant	<i>Ribes nigrum</i>	USA, Arizona, Pinal	111°17'04.21"; 32°48'58.34"	660
S5	Red currant	<i>Ribes rubrum</i>	USA, Arizona, Pinal	111°17'04.21"; 32°48'58.34"	660
S6	Goose berry	<i>Ribes uva-crispa</i>	USA, Arizona, Pinal	111°17'04.21"; 32°48'58.34"	660
S7	Red goji berry	<i>Lycium barbarum</i>	USA, Arizona, Pinal	111°17'04.21"; 32°48'58.34"	660
S8	Goji berry	<i>Lycium infaustum</i>	USA, Arizona, Pinal	111°17'04.21"; 32°48'58.34"	660
S9	Black goji berry	<i>Lycium ruthenicum</i>	USA, Arizona, Pinal	111°17'04.21"; 32°48'58.34"	660
S10	Blueberry	<i>Vaccinium corymbosum</i>	USA, Maine	68°59'05.21"; 45°11'18.34"	426
S11	White mulberry	<i>Morus alba</i> cv. Mashhad	Iran, Karaj, Mohammadshahr	51°00'13.27"; 35°44'23.12"	1228
S12	Red mulberry	<i>Morus rubra</i> cv. Karaj	Iran, Karaj, Mohammadshahr	51°00'13.27"; 35°44'23.12"	1228

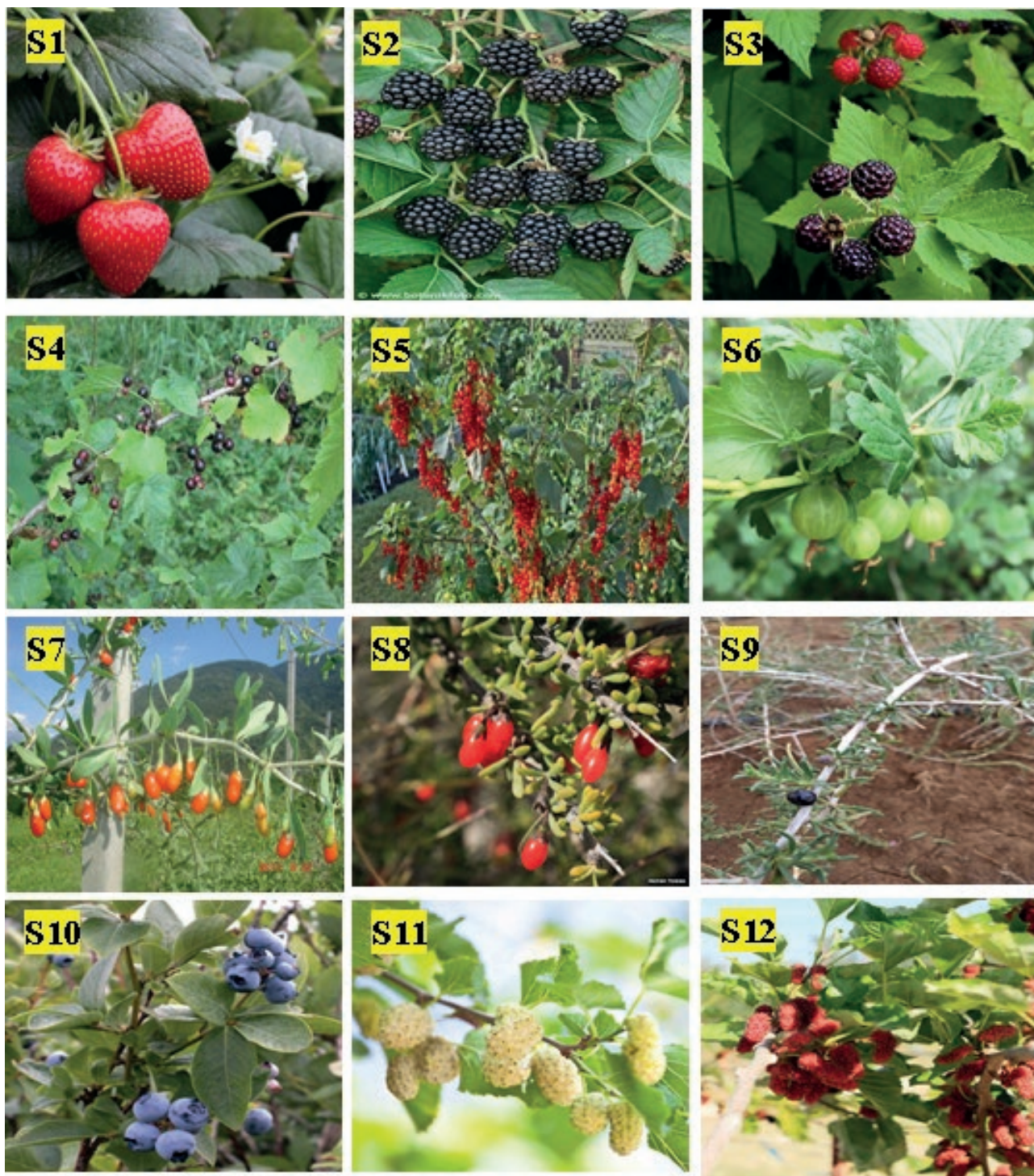


Figure 1. The picture of berry species that studied in this research.

mg l⁻¹). The medium was adjusted to pH = 5.8 and autoclaved for 20 min at 121°C. These plants were grown in a greenhouse at the college of Agriculture, Tarbiat Modares University (TMU). Voucher specimens of the

examined species are preserved in the herbarium of the Research Institute of Forests and Rangelands of Iran (TARI) and the Tarbiat Modares University of Iran (TMU). Also, the images of species are shown in Figure

1. All cultures were maintained in a growth chamber at 25 °C, using a 16 h light/8 h dark photoperiod. The light was supplied, using white fluorescent lamps at an intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Karyomorphology

The best method to study mitosis for preparing a karyotype is root tip meristem tissue, which was used for cytogenetic studies. For the cytological preparations, growing roots about 1 cm long were removed at the appropriate time (when the largest number of cells are in metaphase) and pretreated in 0.002 mol l⁻¹ 8-hydroxyquinoline for 4h at room temperature (RT) in the dark to induce metaphase arrest, followed by washing twice (each for 5 min) in distilled H₂O and fixing in fresh 3:1 (v/v) absolute ethanol: glacial acetic acid for 24 h at 4 °C. The fixed roots were hydrolyzed in 1 M HCl for 7-10 min at 60°C washed two times (each for 5 min) in distilled H₂O and stained in 1% (w/v) aceto-orcein for 3 h or in 4% (w/v) Hematoxylin for 4 h at RT.

Five well-spread monolayer metaphase plates from different individuals were analyzed per species. High-resolution microscopic digital photographs were taken, using an Olympus BX50 microscope (Olympus Optical Co., Tokyo, Japan), equipped with an Olympus DP12 digital camera (Olympus Optical Co.). Eight chromosomal parameters were either measured as long (L) and short (S) arm lengths or calculated as chromosome length (CL), arm ratio (AR; L/S), r-value (S/L), total chromosome volume (TCV = πr^2 CL), where r = average chromosome radius, percentage of total chromosome form (F%), and centromeric index (CI = S/CL). Idiograms were drawn from the mean values, and chromosome types were determined, using the formula of Levan *et al.* (1964). For karyotypic analysis, five parameters, including karyotype total form percentage (TF% = $\Sigma S / \Sigma CL \times 100$), coefficient of variation of total chromosome length ($CV_{CL}\% = (\text{total CL standard deviation} / \text{total CL mean}) \times 100$), mean centromeric asymmetry ($M_{CA} = A \times 100$), where the degree of karyotype asymmetry ($A = [\Sigma(L-S/L+S/n)] \times 100$), Stebbins (1971) asymmetry categories (ST), and Romero-Zarco (1986) indices: intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2), were measured.

Flow cytometric genome size estimation

Relative DNA content was determined, using PI-stained flow cytometry. Young leaves of the analyzed individuals and a reference standard (either *Solanum*

lycopersicum; 2C = 1.96 pg., *Petroselinum crispum*; 2C = 4.45 pg., or *Zea mays*; 2C = 5.43 pg) were co-chopped with a razor blade in a glass petri dish, containing 1 ml of ice-cold WPB buffer (Woody Plant Buffer; 0.02 M Tris-HCl, 4 mM MgCl₂.6H₂O, 2 mM EDTA Na₂.2H₂O, 86 mM NaCl, 10 mM Sodium metabisulfite, 1% PVP-10, and 1% (v/v) Triton X-100, pH 7.5) (Loureiro *et al.* 2007). The crude nuclei suspension was filtered through a 50- μm nylon mesh. RNase and propidium iodide (each 50 $\mu\text{g ml}^{-1}$) was then added. After incubation for two min at RT, the relative fluorescence intensity of nuclei was analyzed. At least 5000 nuclei were analyzed in each measurement with CV% (coefficient of variation; %) values below 5.0%. Subjects were randomly selected for the flow cytometric analysis. The DNA amount of a sample was calculated based on the values of the G1 peak means (Doležel *et al.* 2003, 2007; Doležel and Bartoš 2005) as follows:

Sample 2Cx DNA (pg) = (Sample G1 peak mean/Standard G1 peak mean) \times Standard 2C DNA (pg).

The obtained data were initially checked for the normality test and analyzed, using SPSS (Version 22) and Minitab 17. The karyotypic, flow cytometric, and phytochemical content data were first tested for the normality and then analyzed based on a completely randomized design (CRD) with 5, 3, and 3 replications, respectively. The results were statistically evaluated by analysis of variance (ANOVA) and expressed as mean. Means comparisons were performed, using the LSD test with SPSS; differences were considered statistically significant at $P \leq 0.01$ and $P \leq 0.05$. Linear regression analysis was carried out to find out the relationship between monoploid 2Cx DNA values and some traits, using Minitab 17.

Chromatographic analysis

The method used to determine total polyphenol content (TPC) was based on Folin-Ciocalteu phenol reagent and spectrophotometric determination at 765 nm. The method used to determine the total flavonoid content (TFC) was based on aluminum chloride and spectrophotometric determination at 420 nm. Antioxidant activity in the berries fruit pulp was evaluated by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Laczkó-Zöld *et al.* 2018).

Total Antioxidant Capacity (TAC)

The scavenging activity for DPPH radicals was determined using spectrophotometric analysis accord-

ing to a modified method of Lalegani *et al.* (2018). Briefly 100 μ l sample solution was added to 900 μ l of 0.1 M DPPH solution and mixed thoroughly at RT. Absorbance at 517 nm was determined after 30 min.

Total Phenolic Content (TPC)

The TPC was estimated, using the Folin- Ciocalteu colorimetric method described by Barreca *et al.* (2016). A 20 μ l of methanolic extract was added to 2 ml of deionized water, and 100 μ l of dilutions Folin- Ciocalteu was then added. After 1-8 min (average 5 min), 300 μ l of Sodium carbonate 7% (w/v) was added to it, and the absorbance was measured in the dark at 765 nm after incubation for 2 h at RT. Quantification was done based on the standard curve of gallic acid. Results were expressed as equivalent of the gallic acid (GAE), i.e., mg gallic acid g^{-1} DW.

Total Flavonoid Content (TFC)

The TFC was estimated, using the aluminum chloride method described by Barreca *et al.* (2016). In this method, a 600 μ l of methanolic extract was added to 600 μ l of 2% (w/v) Aluminum chloride, and after 10 min, the absorbance was measured at 420 nm. Quantification was done based on the standard curve of quercetin. The TFC was calculated and expressed as quercetin equivalents, i.e., mg quercetin g^{-1} DW.

RESULTS

Karyomorphologic analysis

In the current study, one chromosome type “m” formed karyotype formulas of “14m” for *Fragaria vesca* subsp. *vesca* (S1), *Rubus fruticosus* cv. Qaemshahr (S2), and *Rubus occidentalis* cv. Qaemshahr (S3), “16m” for *Ribes nigrum* (S4), *Ribes rubrum* (S5), and *Ribes uva-*

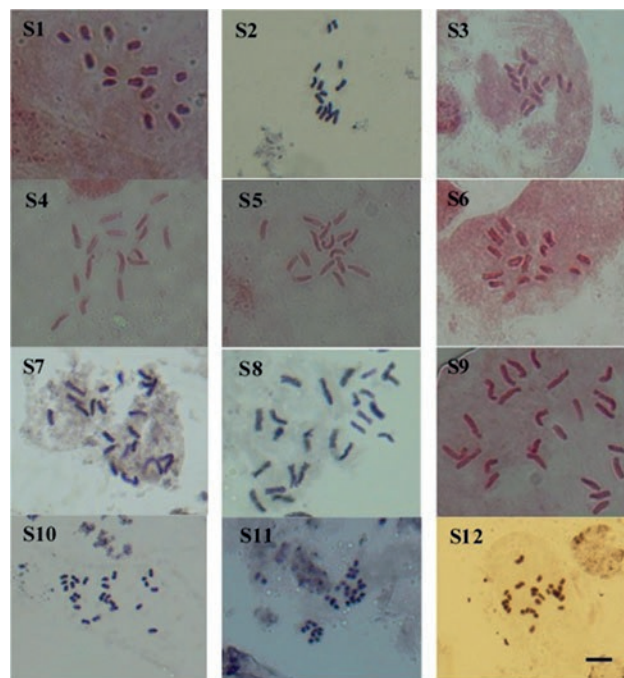


Figure 2. Somatic chromosomes of 12 berry species. Scale bar = 5 μ m.

crispa (S6), “24m” for *Lycium barbarum* (S7), *Lycium infaustum* (S8), *Lycium ruthenicum* (S9), and *Vaccinium corymbosum* (S10), and “28m” for S11 and S12 (*Morus alba* cv. Mashhad (S11), *Morus rubra* cv. Karaj (S12). The base chromosome number in *Fragaria vesca* subsp. *vesca*, *Rubus fruticosus* cv. Qaemshahr, *Rubus occidentalis* cv. Qaemshahr; is $x = 7$; *Ribes nigrum*, *Ribes rubrum*, *Ribes uva-crispa* $x = 8$; *Lycium barbarum*, *Lycium infaustum*, *Lycium ruthenicum*, *Vaccinium corymbosum* $x = 12$; *Morus alba* cv. Mashhad, *Morus rubra* cv. Karaj $x = 14$, and all of these species are diploid with $2n = 14$, 16, 24, and 28.

ANOVA shows significant ($P < 0.01$) differences between the berries species for the most studied chromosomal parameters (Table 2). Karyotypes of somatic complement and the idiograms of the haploid comple-

Table 2. ANOVA for chromosomal parameters of berry species.

S.O.V.	DF	MS							
		S	L	CL	AR	r-value	F%	TCV	CI
Species	11	7.413**	8.825**	20.685**	0.128**	0.128**	23.661**	49.778**	0.199**
Error	593	0.039	0.046	0.11	0.017	0.017	0.121	0.422	0.057
Total	604								

**Significant difference at $P < 0.01$

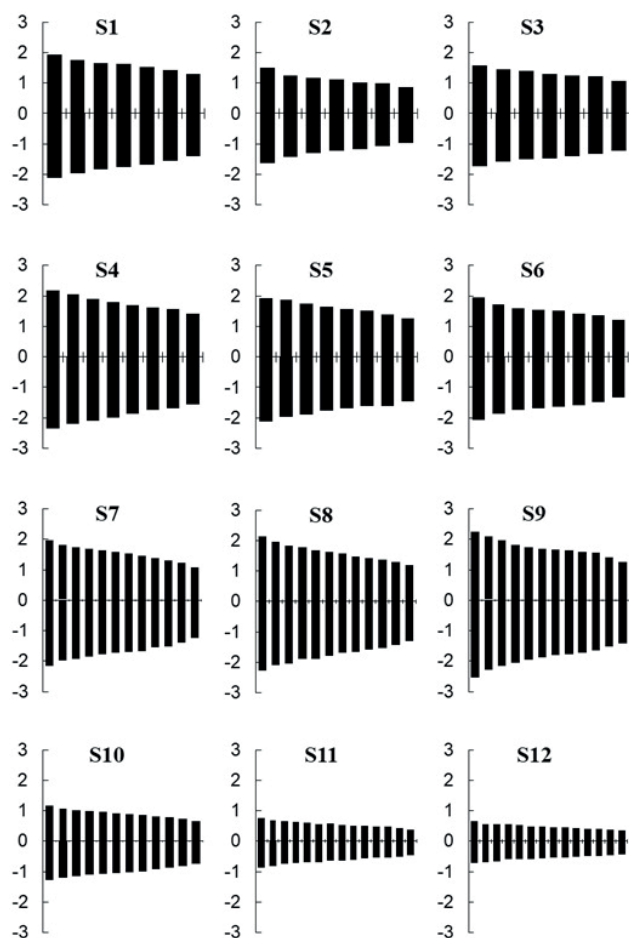


Figure 3. Idiograms of haploid chromosomes of 12 berry species.

ment of studied berries are demonstrated in Figures 2 and 3, respectively. The mean value of chromosome length (CL) was 2.83 μm , varying from 1.06 μm (*Morus rubra* cv. Karaj; S12) to 3.75 μm (*Ribes nigrum*; S4). The mean TCV was 1.6 μm , ranging from 0.10 μm (S10) to 6.19 μm (S1). The mean CI of the complement varied from 46% (S12) to 51% (S5). The studied berry species showed different symmetrical groups according to various karyotypic symmetrical indices. For instance, the highest value of total form percentage of karyotype (TF%) was detected in S5 (50.51%; Table 3; the most symmetric), and the lowest value was identified in S12 (46.28%; the most asymmetric). The highest and the lowest values of coefficient of variation ($CV_{CL}\%$) were identified on S11 (18.03%; the most asymmetric) and S3 (11.98%; the most symmetric), respectively. On the other hand, karyotypes of all these species were classified in 1A class of Stebbins classification (Stebbins 1971; Table 3). The basic chromosome number for these ber-

Table 3. Karyotypic symmetry formula and flow cytometric genome size of 12 berry species. Total form percentage of karyotype (TF%), mean centromeric asymmetry ($M_{CA}\%$), coefficient of variation of chromosome length ($CV_{CL}\%$), karyotype formula (KF), Stebbins asymmetry categories (ST), intrachromosome asymmetry index (A1), interchromosome asymmetry index (A2), m: metacentric, M: metacentric. Means with the same symbol letter in the “2Cx DNA (pg)” column are not significantly different ($P > 0.01$), using LSD test.

Species	Asymmetry indices (Romero-Zarco, 1986)		ST	KF	$CV_{CL}\%$	$M_{CA}\%$	TF%	2Cx DNA (pg)
	A1	A2						
S1	0.46	0.13	1A	14m	13.38	4.37	47.79	2.10 ^d
S2	0.47	0.18	1A	14m	17.71	4.91	47.59	1.55 ^g
S3	0.47	0.12	1A	14m	11.98	4.73	47.66	0.86 ⁱ
S4	0.39	0.14	1A	16m	14.21	4.31	47.86	1.86 ^e
S5	0.33	0.16	1A	16m	16.17	4.54	50.51	1.94 ^e
S6	0.39	0.15	1A	16m	14.61	4.30	47.98	1.94 ^e
S7	0.10	0.16	1A	24m	15.82	5.35	47.38	3.93 ^b
S8	0.09	0.17	1A	24m	16.65	4.73	47.68	3.82 ^b
S9	0.09	0.16	1A	24m	16.51	4.75	47.60	5.15 ^a
S10	0.11	0.16	1A	24m	15.63	5.91	47.07	2.53 ^c
S11	0.00	0.18	1A	28m	18.03	7.31	46.37	1.65 ^f
S12	0.00	0.16	1A	28m	16.05	7.58	46.28	1.18 ^h

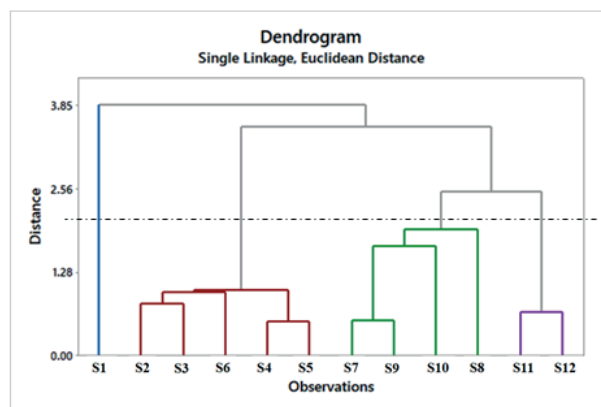


Figure 4. Dendrogram shows relationships of similarity among studied 12 berry species related to all chromosomal characteristics, constructed using Euclidean distance and Single method; cophenetic correlation $r = 0.75$.

three groups. To determine total variation in berries and parameters quota in total variation, the principal component analysis (PCA) of the karyotypic parameters was performed. The single dendrogram constructed based on karyotype similarities (Figure 3) shows four major clus-

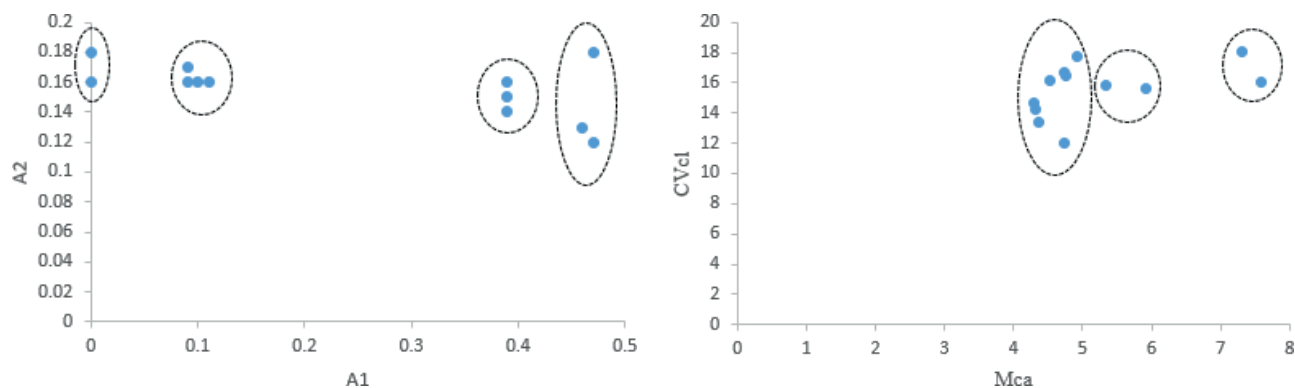


Figure 5. Scatter diagram of 12 berry species: Romero-Zarco asymmetry indices (a), CVCL and MCA (b).

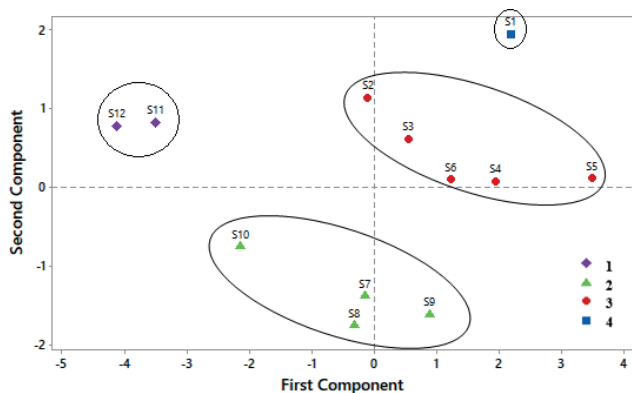


Figure 6. Diagram resulted from principal components analysis 1 (highly related to the position of centromere) and 2 (strongly related to the length of the complements) of studied berry species.

ters. The first cluster consists of S11 and S12, the second cluster consists of S7-S10, and the third cluster consists of S2-S6, while S1 is separated within the fourth cluster.

Nuclear genome size estimation

The resultant flow cytometric data were first tested for normality test and then analyzed according to a completely randomized design (CRD) with 3 replicate cells. The monoploid genome size ($2Cx$ DNA) of these twelve species (*Fragaria vesca* subsp. *vesca*, *Rubus fruticosus* cv. Qaemshahr, *Rubus occidentalis* cv. Qaemshahr, *Ribes nigrum*, *Ribes rubrum*, *Ribes uva-crispa*, *Lycium barbarum*, *Lycium infaustum*, *Lycium ruthenicum*, *Vaccinium corymbosum*, *Morus alba* cv. Mashhad, *Morus rubra* cv. Karaj) were 2.1, 1.55, 0.68, 1.86, 1.94, 1.94, 3.93, 3.82, 5.15, 2.53, 1.65, and 1.18 pg, respectively (Table 3). The differences in nuclear DNA contents among these analyzed species were statistically significant ($P < 0.01$). The highest value was detected

in *Lycium ruthenicum* (S9, 5.15 pg), while *Rubus occidentalis* cv. Qaemshahr (S3) was the lowest (0.68 pg), and the mean total value was 2.36 pg (Table 3).

The histograms obtained for analyzing nuclear DNA contained two peaks (Figure 6). The left peaks in S7 to S10 refer to the known samples reference standard, and the right peaks to the unknown samples. In other species, the peaks on the left refer to unknown specimens, and the peaks on the right refer to the reference standard of known specimens. Young leaves of berries spe-

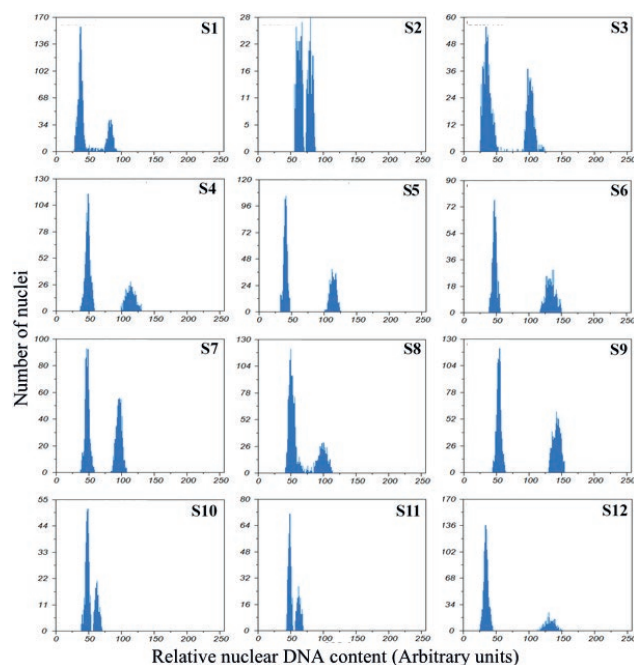


Figure 7. Histograms of relative fluorescence intensity were obtained, using WPB isolation buffer after simultaneous analysis of nuclei isolated from standard plant and berry species plants. The mean channel number and coefficient of variation (CV%) value of each peak are also given.

cies contain many cytosolic compounds such as phenolic acids and flavonoids, which can interfere with the fluorescent staining of nuclear DNA. To compensate this problem, a new isolation buffer, WPB, was developed to analyze problematic or woody species because the Sodium metabisulfite and PVP-10 in WPB act as reducing and binding agents to prevent the action of interfering phenols and secondary metabolites. This buffer was found to be suitable for the analysis of *Lycium* species, as evidenced by the CV of DNA peak and flow cytometric histograms of relative fluorescence intensity. Phylogenetic analysis revealed that *Lycium* species are included in a monophyletic group, indicating a very close evolutionary relationship. This study discovered significant differences in the genome size among these three species (*L. barbarum*, *L. ruthenicum*, and *L. infaustum*). It was also shown in *Ribes*, *Rubus*, and *Morus* species. There is excellent genome size variation between species. It has been proposed that having a large genome has direct physiological consequences for plants, such as earlier flowering time, larger stomata size, and longer life cycles than small genomes. The flowering time may be partly affected by differences in DNA content.

Phytochemical studies

ANOVA shows significant ($P < 0.01$) differences between the berries species for the three studied phy-

tochemical traits, including total antioxidant capacity (TAC), total phenol content (TPC), and total flavonoid content (TFC). The mean comparisons are shown in Table 4, using LSD test at 0.01 probability level. The average total antioxidant capacity (TAC; Table 4) of berries was $32.79 \mu\text{mol g}^{-1}$, ranging from $6.92 \mu\text{mol g}^{-1}$ (S1; *Fragaria vesca* subsp. *vesca*) to $63.84 \mu\text{mol g}^{-1}$ (S3; *Rubus occidentalis* cv. Qaemshahr). The average total phenol content (TPC; Table 4) of berries was $4.98 \mu\text{mol g}^{-1}$, ranging from $3.08 \mu\text{mol g}^{-1}$ (S12; *Morus rubra* cv. Karaj) to $8.61 \mu\text{mol g}^{-1}$ (S9; *Lycium ruthenicum*). The average total flavonoid content (TFC; Table 4) of berries was $5.18 \mu\text{mol g}^{-1}$, ranging from $2.43 \mu\text{mol g}^{-1}$ (S12; *Morus rubra* cv. Karaj) to $10.63 \mu\text{mol g}^{-1}$ (S9; *Lycium ruthenicum*). Black raspberry (S3; *Rubus occidentalis* cv. Qaemshahr) in terms of all three phytochemical traits, had the highest score among all berry species.

Interestingly, the level of antioxidant activity of black raspberry (S3; *Rubus occidentalis* cv. Qaemshahr) had statistically significant difference among all studied berry species in the present study at a probability level of 1%. For example, it had 6.6-fold increase over strawberry (S1; *Fragaria vesca* subsp. *vesca*) and a 24% increase over Black currant (S4; *Ribes nigrum*). In the next positions, Goose berry (S6; *Ribes uva-crispa*) and Black currant (S4; *Ribes nigrum*) had the highest values, respectively. Moreover, strawberry (S1; *Fragaria vesca* subsp. *vesca*) and Goji berry (S8; *Lycium infaustum*) had the lowest one.

DISCUSSION

Our results provided the basic genetic, genomic and phytochemical information for these species, which are helpful for the construction of genetic and physical maps and whole-genome sequencing in the future and provide scientific insight into the phenolic and antioxidant functions to consumers and the nutraceutical industry.

For the first time, some of these species' chromosome number and karyotype were determined in Iran. Chromosome numbers $2n = 2x = 14, 16, 24,$ and 28 in these berry species are consistent with other reports. Differences in the karyotype formula of these species, which are geographically, climatically, and habitually different from each other, indicating the existence of chromosomal structural changes in the process of karyotype evolution and species formation in these species. It varies according to geographical and climatic conditions. Structural changes in the speciation process of these species can be changed such as displacements, inversions, and other structural changes. According to Stebbins

Table 4. Information obtained from phytochemical evaluation of studied 12 berry species. Total Antioxidant Capacity (TAC), Total Flavonoid Content (TFC), Total Phenol Content (TPC). In each column, means with the same symbol letter are not significantly different ($P > 0.01$), using LSD test.

Species	TAC ($\mu\text{mol g}^{-1}$)	TFC (mg g^{-1})	TPC (mg g^{-1})
S1	9.62 ^h	2.47 ^f	3.08 ^f
S2	38.40 ^c	5.40 ^c	5.75 ^b
S3	63.84 ^a	9.60 ^a	7.92 ^a
S4	51.57 ^b	6.50 ^b	5.82 ^b
S5	24.56 ^e	3.43 ^d	3.73 ^d
S6	52.19 ^b	9.23 ^a	7.68 ^a
S7	25.06 ^e	3.45 ^d	4.07 ^c
S8	16.38 ^g	2.57 ^{ef}	3.17 ^{ef}
S9	34.95 ^c	10.63 ^a	8.61 ^a
S10	27.50 ^{de}	2.77 ^e	3.28 ^e
S11	29.11 ^d	3.70 ^d	3.59 ^d
S12	20.32 ^f	2.43 ^f	3.08 ^f
Average (range)	32.79 (9.62-63.84)	5.18 (2.43-10.63)	4.98 (3.08-8.61)

(1971), cytogenetic studies can be used to understand better the relationships between species and different populations of a species and to orient the evolutionary tendencies of plants. Each of these plants shows a unique adaptation to the environment in which they grow. As this compromise increases, new varieties or species may appear in plant communities. This study developed an important tool to assess berries' chemical composition and bioactivity, using different chromatographic methods for its fruits' comprehensive authentication and quality control. In the present study, all chromosomal parameters were significantly different. Differences between species for all karyological parameters confirm the intraspecific chromosomal variations. These results are in agreement with those reported by Chen *et al.* (2013). In the current study, one chromosome type ("m") formed karyotype formulas of "14m" for S1-S3 (*Fragaria vesca* subsp. *vesca*, *Rubus fruticosus* cv. Qaemshahr, *Rubus occidentalis* cv. Qaemshahr), "16m" for S4-S6 (*Ribes nigrum*, *Ribes rubrum*, *Ribes uva-crispa*), "24m" for S7-S10 (*Lycium barbarum*, *Lycium infaustum*, *Lycium ruthenicum*, *Vaccinium corymbosum*), and "28m" for S11 and S12 (*Morus alba* cv. Mashhad, *Morus rubra* cv. Karaj). Similar to our findings, Chen *et al.* (2013) reported the same one chromosome type in *Lycium* species. In the present study, the average total length of chromosomes (CL) in the studied species is 2.83 μm . Karyotype symmetry was more indicative of evolutionary traits in the studied berries species, regardless of ploidy levels. To determine the karyotypic symmetry, several properties were examined in 12 species. Based on TF%, S6 with the highest value (47.98%) has the most symmetric, and S12 with the lowest value (46.28%) has the most asymmetric karyotype. Gradual changes and changes in TF% values may be due to chromosomal abnormalities. Structural changes in chromosome morphology probably due to chromosome duplication or translocation (exchange and displacement) between chromosomes (Das and Teng 1998). According to the $CV_{CL}\%$ index, S3 (11.98%) has the most symmetrical karyotype and S11 (18.03%) has the most asymmetric karyotype among other species. Overall, the coefficient of variation in the sample was low due to the metacentric nature of most chromosomes, indicating the symmetry and the uniformity of these karyotypes. In general, it can be concluded, species that are more evolutionarily advanced have variations in chromosome type and size and are therefore asymmetrically karyotypically. This asymmetry is exacerbated by the displacement of chromosomal fragments. Karyotypic dissimilarity in terms of karyotype symmetry measurement parameters can lead to failure in reproduction and inadequate seed production in the offspring while pre-

venting successful intraspecific crosses. In other words, the results of such crosses may be somewhat sterile. In the study of Chen *et al.* (2013) on the genus *Lycium*, the chromosomal base number for red goji berry (S7; *L. barbarum*) and black goji berry (S9; *L. ruthenicum*) was reported to be 12, which is in complete agreement with the findings of the present study (Table 3). Costich *et al.* (1993) worked on the blueberry plant (S10; *Vaccinium corymbosum*) showed that the chromosomal base number was 12, which is consistent with the findings of the current study. For the genus *Ribes*, the base chromosome number 8 was reported by Chiche *et al.* (2003), which is matched for the three different species of this genus (S4; *R. nigrum*, S5; *Ribes rubrum*, S6; *R. uva-crispa*) in the present study.

On the other hand, karyotypes of all species were classified in 1A (Table 3). For more detailed studies of asymmetry, Romero-Zarco (1986) indices of A1 and A2 were also calculated. The scatter diagram of these indices (Figure 4) presents four species groups. According to the analyzed asymmetry indices, S3 was demonstrated as the most symmetric species, while S2 and S11 were among the most asymmetric species. It has been suggested that karyotypes with more asymmetry have a derived status in comparison to those with more symmetrical morphology (Lakshmi *et al.* 1984). For example, differences in chromosome length (CL) may indicate the occurrence of cyclic changes in genome size during the diversification of the genus. Thus, the study of asymmetry indices and variation in genome size is a valuable means for the establishment of the evolutionary relationship between the species and the origin of diversification of the populations (Karimzadeh *et al.* 2011).

To determine the total diversity in the population and the quota of the parameters in the total diversity, principal component analysis (PCA) of the karyotypic parameters was performed. It shows that the first two main components make up 82% of the cumulative changes and they are predicted in a two-dimensional graph (Figure 5). The single dendrogram constructed on the basis of karyotype similarities (Figure 3) shows four major clusters. The first cluster consists of S11 and S12, the second cluster consists of S7-S10, and the third cluster consists of S2-S6, while S1 is separated within the fourth cluster. The arrangement of PCA species from this experiment is fully consistent with the analysis obtained by single grouping analysis. Therefore, the results of this study proposed that species within a cluster have the most homology in chromosomal variations. For this purpose, a cross between S7 and S8 or S9 is suggested because they have the most similarity in their chromosomal characteristics.

The mean monoploid 2Cx DNA value was 2.35 pg in the studied species, verifying intraspecific genome size variation. On the other hand, the monoploid genome size (1Cx DNA) varied from 420.54 Mbp (S3; *Rubus occidentalis* cv. Qaemshahr) to 2518.35 Mbp (S9; *Lycium ruthenicum*). According to the results (Chen *et al.* 2013), red goji berry (S7; *Lycium barbarum*) and black goji berry (S9; *Lycium ruthenicum*) had 2Cx DNA values of 4.35 pg and 5.45 pg, respectively. In the present study, the genome size for the those was 3.93 pg and 5.15 pg, correspondingly, which is almost in agreement with the findings of the previous report. The existence of some deviation from this species may be due to the inter-species and intersex diversity. Knowing genome size may be useful in genome research and studies of relationships between DNA content, physiology, and plant ecology (Thiem and Sliwinska 2003). Reports of 2Cx DNA in some berry species have been published, including S3; *Rubus occidentalis* = 0.60 pg, S5; *Ribes rubrum* = 1.94 pg, and S6; *Ribes uva-crispa* = 1.88 pg (Meng and Finn 2002; Chiche *et al.* 2003). In the present study (Table 3), the monoploid genome size (2Cx DNA) for the latter three species is 0.68 pg, 1.86 pg, and 1.94 pg, respectively, which is in complete agreement with the findings of the two previous reports. In the present study, the existence of a statistically significant difference in the monoploid genome size indicates the interspecific and intersex diversity among berry species. On the other hand, the correlation between 2Cx DNA with environmental conditions (longitude, latitude, and altitude), chromosome length, and the chromosome number showed that 2Cx DNA in the studied berry species had a significant correlation with the chromosome length and chromosome number, and there were no significant correlations between 2Cx DNA with environmental conditions. Hence, it is concluded that the genome size of berries species is independent of changes in environmental conditions.

Antioxidant properties estimated by antioxidant assays (DPPH) showed significant differences among different concentrations. Evaluation of total polyphenol content in samples is a widely used method to determine the number of antioxidants in the samples. A rapid, simple, and inexpensive method to measure the antioxidant capacity of food involves the use of the free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH radicals are frequently utilized in antioxidant studies. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and evaluate the antioxidant activity of foods. Antioxidants in a sample can scavenge the DPPH radicals. A gradual reduction in absorbance is observed, indicating that DPPH radicals

are being scavenged. Through adding samples, which are rich in antioxidants, to a DPPH solution. Therefore, the percentages we have presented pertain to DPPH radical scavenging capacity, which is directly proportional to antioxidant capacity. The total phenolic content of dry fruits showed a wide range, with values ranging from 3.08 mg GAE/g (S1; *Fragaria vesca* subsp. *vesca*) to 8.61 mg GAE/g (S9; *Lycium ruthenicum*) shown in Table 4. By comparing these data, it can be concluded that the S3 (*Rubus occidentalis* cv. Qaemshahr) has a high amount of antioxidants. The total flavonoid content (TFC) of dried fruits showed a wide range, with values ranging from 2.43 g QE/g (S12; *Morus rubra* cv. Karaj) to 10.63 g QE/g (S9; *Lycium ruthenicum*) as shown in Table 4.

Based on the above studies and considering the diversity of these 12 species in terms of cytology and phytochemistry, it is concluded that some of these species are prone to enter the daily food basket. Black raspberry (S3; *Rubus occidentalis* cv. Qaemshahr) had the highest value in terms of all three phytochemical traits, followed by goose berry (S6; *Ribes uva-crispa*) and black currant (S4; *Ribes nigrum*), respectively. Strawberry (S1; *Fragaria vesca* subsp. *vesca*) and red mulberry (S12; *Morus rubra* cv. Karaj) had the lowest value. We report that black raspberry (S3; *Rubus occidentalis* cv. Qaemshahr) is a remarkable source of antioxidant compounds compared to other fruits. Hence, research supports deep-colored fruits as potent antioxidant sources. Berries and dried fruit compose a relatively small part of the average diet, but they are important antioxidant sources. Highly pigmented berries have the highest antioxidant activity. Such fruits are rich in antioxidant compounds that are known for their enhanced stability and bioaccessibility. Based on our findings and the cited literatures, it can be suggested that black raspberries are among the fruits that provide antioxidants. In the study of Islam *et al.* (2017) on genus *Lycium*, the mean of total phenol content, total flavonoids content, and antioxidant activity for red goji berries (S7; *Lycium barbarum*) were measured as 3.16 mg g⁻¹, 2.83 mg g⁻¹, and 16.65 μmol g⁻¹ respectively. These values were 8.33 mg g⁻¹, 11.03 mg g⁻¹, and 34.28 μmol g⁻¹ respectively for black goji berry (S9; *Lycium ruthenicum*). In the present study (Table 4), these values were 4.07 mg g⁻¹, 3.45 mg g⁻¹, and 25.06 μmol g⁻¹ respectively, for S7 with a significant increase of 29%, 22%, and 50% in all three cases, respectively, compared to the previous report of red goji berry (S7; *Lycium barbarum*). Also for S9, these values are reported 8.61 mg g⁻¹, 10.63 mg g⁻¹, and 34.95 μmol g⁻¹, respectively, which is almost equal to the previous report for (S9; *Lycium ruthenicum*). In the study of Mustafa Ahmed *et al.* (2022) on genus *Vaccinium*, the mean of total phenol

Table 5. Correlation coefficients between phytochemical traits of 12 berry species with chromosome number, chromosome length, the monoploid genome size (2Cx DNA), and environmental conditions

Phytochemical traits	Latitude (N)	Longitude (E)	Altitude (m)	Chromosome (No.)	CL (μm)	2Cx DNA (pg)
Total phenol content (mg g^{-1})	-0.28 ^{ns}	0.29 ^{ns}	-0.30 ^{ns}	-0.38*	0.40*	0.10 ^{ns}
Total flavonoid content (mg g^{-1})	-0.29 ^{ns}	0.29 ^{ns}	-0.25 ^{ns}	-0.34*	0.39*	0.09 ^{ns}
Antioxidant capacity (DPPH) ($\mu\text{mol g}^{-1}$)	-0.09 ^{ns}	0.10 ^{ns}	-0.19 ^{ns}	-0.44*	0.08 ^{ns}	-0.34*

^{ns}, *, **: Non significant ($P > 0.05$), significant differences ($P < 0.05$) and ($P < 0.01$), respectively.

content, total flavonoid content, and antioxidant capacity were reported as 3.73 mg g^{-1} , 2.57 mg g^{-1} , and $17.67 \mu\text{mol g}^{-1}$, respectively. In the present study (Table 4), these values were 3.28 mg g^{-1} , 2.77 mg g^{-1} , and $27.50 \mu\text{mol g}^{-1}$, respectively which are almost in agreement with those in the previous report.

In the current study, the correlation was carried out between phytochemical traits with chromosome number, chromosome length, the monoploid genome size (2Cx DNA), and environmental conditions (latitude and altitude). The results showed no significant correlation between phytochemical traits of species with environmental conditions, but there was a significant correlation between phytochemical traits with chromosome number, antioxidant activity with genome size, total phenol with chromosome length, and total flavonoid with chromosome length (Table 5). From these results, it is inferred that phytochemical traits in the studied species are independent of changes in environmental conditions.

For traits that had a significant correlation between them in the table above, linear regression analysis was performed (Figure 7).

Figure 7 shows that the monoploid genome size (2Cx DNA) has a direct linear relationship with chromosome length and chromosome number of berry plants (Figures a, and b). Antioxidant has an inverse linear relationship with the genome size and chromosome number (Figures c, and d). It means that berries with fewer chromosomes and smaller genome sizes produce more antioxidants. Phenol and flavonoid have an inverse linear relationship with the chromosome number (Figures e, and f), but a direct relationship with the chromosome length (Figures g, and h).

For further study, the correlation between genome size and chromosome length for four species (S7, S8, S9, S10) with the same chromosome number ($2n = 2x = 24$) was also calculated and was significant at the 1% probability level ($r = 0.89^{**}$). Therefore, Figure 8 shows the relationship of the direct linear regression between the monoploid genome size and chromosome length for the four species mentioned above.

In another study, the effect of different ploidy levels on the quantity and quality of essential oils of different species of berry was investigated and their genetic modification was provided. It is suggested that these berry species should be compared with other species in Iran in terms of cytogenetics and phytochemistry. It is suggested that due to significant differences in genome size and morphology, further research should pin-point rDNA (5S and 45S) sites and additional repetitive DNA elements, using fluorescence *in situ* hybridization (FISH) to better uncover the processes involved in the chromosome evolution of these twelve berry species.

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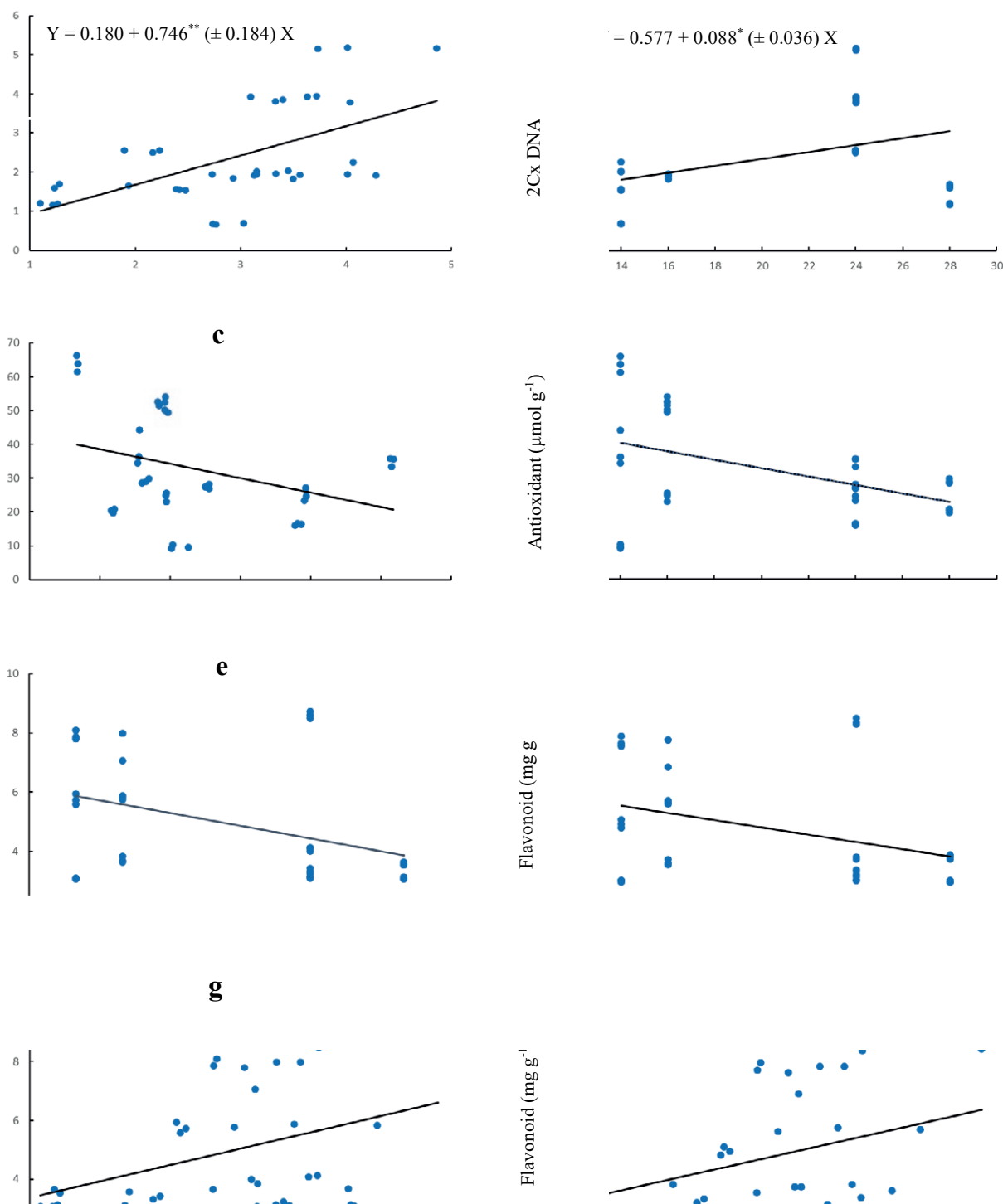


Figure 8. Diagrams of linear regression relationships between different traits of studied berry plants

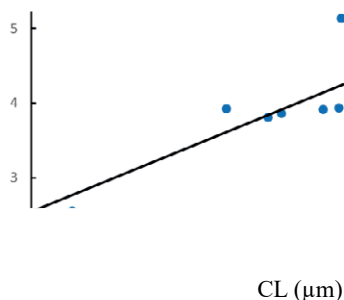


Figure 9. Diagram of the linear regression relationship between genome size and chromosome length for four species (S7-S10) with the same chromosome number ($2n = 2x = 24$).

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