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Research Article

A comparative investigation of nickel hyperaccumulation and local adaptation in some of the *Alyssum* species

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Abstract

The relationship between the physiological nickel hyperaccumulation character, endemism in serpentine soils, tolerance indices, and karyotype differences of some accessions of nickel hyperaccumulator and non-accumulator of the genus *Alyssum* from serpentine and non-serpentine soils was investigated. Karyotypes were obtained from prometaphase and metaphase analysis maps by root meristem chromosomal staining. Physiological responses to nickel in the medium, including catalase activity as an indicator of iron accessibility, and photosynthetic pigments were measured. Karyotype of diploid species, 2n=2x=16, 20 and 22, was determined that the type of chromosomes are metacentric, submetacentric, acrocentric and telocentric. The results of chromosomes' character analysis, such as chromosome asymmetry coefficient, showed a significant difference between these species in terms of various characters. The examination of the morphology of chromosomes revealed that species with more diverse chromosome types and superior chromosomal characteristics, such as secondary constriction, having the least metacentric chromosome, low centromeric index, and more asymmetric karyotype, were not nickel hyperaccumulators. Other data showed a decreased tolerance capacity of the non-serpentine plants via adaptation to normal soils. The data showed a correlation between nickel hyperaccumulation and primitive karyotype features in the *Alyssum* genus, and adaptations to normal soils could have diminished the intensity of the nickel hyperaccumulation trait.

Keywords: Adaptation, *Alyssum*, Brassicaceae, Karyotype, Metal accumulation, Nickel, Serpentine

Introduction

Studying chromosome structures, including chromosome number, type, and special features such as secondary constrictions, is very important for finding interrelationships of taxa and their common physiological and metabolic characters. The most significant discoveries about chromosomes' organization and dynamics, and the effect of these two factors on the occurrence of genes in plants of the *Brassicaceae*, are related to the investigation of the chromosomal arrangement of *Arabidopsis thaliana* in the interphase (Fang and Spector, 2005). It has been determined that the organization of the interphase chromosome is controlled by genetic and environmental factors (Wang, 2011).

Alyssum is a genus of the *Brassicaceae* with species having the nickel hyperaccumulation character, and new molecular phylogenetic studies using different molecular markers such as AFLP have helped to reveal the evolutionary dynamics of nickel hyperaccumulation ability in *Alyssum* and *Alysseae* (Coppi *et al.,* 2020; Li *et al.,* 2015; Resetnik *et al.,* 2013; Cecchi *et al.,* 2010). Among the groups of angiosperms, *Alyssum* (*Odontarrhena*) is one of the most important groups in terms of taxonomic diversity and distribution range, including the most nickel-accumulating species in the European Mediterranean region (Gabbrielli *et al.,* 1991; Baker and Brooks, 1989). According to findings, species of this group that grow in serpentine soils, can uptake and store more than 1000 µg of nickel in the dry weight of the leaf tissue without toxicity symptoms (Kramer, 2010; Baker and Brooks, 1989). Some physiological aspects related to metal hyperaccumulation character are known in different species. For example, it has been determined that the free histidine amino acid in root cells plays a role in resistance and probably the transfer of nickel across the root cells from the epidermis to the xylem elements (Kerkeb and Kramer, 2003). *Alyssum* genus has a number of enzymes and antioxidant molecules that are quite effective and active, which protect the plant against oxidative damage, especially since accumulation is mainly in active parts such as emerging leaves and even at the end of growing stems. With increase in soil

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pH, the accumulation of nickel also increases in seedlings, which is in contrast to other plants' reactions in taking up metals, which do better under acidic pH conditions and may show physiological and genetic adaptations to the edaphic conditions (Whiting *et al.,* 2003; Chaney *et al.,* 1997; Nicks and Chambers, 1995; Homer *et al.,* 1991). The adaptation of plants to the harsh and unfavorable biological conditions that exist in serpentine soils, in various studies, is attributed to a kind of adaptation and the emergence of new alleles in the populations settled on these soils. Since serpentine soils are essentially separated and scattered in small patches, different researchers do not evaluate the occurrence of adaptive characters as monotony, in these habitats (Konecna *et al.,* 2020). The most important characters that should develop in adaptation to these habitats are drought resistance, effective nutrients' absorption such as nitrogen, calcium, and phosphorus, and resistance (tolerance) to heavy metals such as nickel. Researchers consider the appearance of metal hyperaccumulation character as a resistance to large amounts of metal in the plants' growth medium (Konecna *et al.,* 2020; Stein *et al.,* 2017).

Understanding the dynamic adaptation to the environments is achieved through investigation of wide ranges, including molecular (chromosomes, genes, and alleles), physiological (functional biology), biochemical (enzymatic and adaptive chemicals), anatomical and ecological aspects of the required changes that could adapt to serpentine soils. Several studies and reviews have revealed some details of this phenomenon (e.g. Navarrete Gutierrez *et al*., 2024; Meindl *et al.,* 2021; Konecna *et al.,* 2020; Ghasemi *et al.,* 2020; Kramer, 2010; Kazakou *et al.,* 2008; Bradshaw, 2005; Brady *et al*., 2005). Although the patterns of chromosomal evolution are extensively diverse and is influenced by many factors (Eichler and Sankoff, 2003), it is generally believed that some changes in chromosomes and karyotypes indicate more evolved and advanced features of the organism than its ancestors. For example, asymmetric karyotype is considered more advanced than symmetric karyotype and chromosome rearrangements can happen while adapting to new environments (Lysak and Weiss-Schneeweiss, 2021; Guerrero and Kirkpatrick, 2014; De Storme and Mason, 2014).

In addition, according to the information available about the nickel hyperaccumulation ability in these plants, a relationship between this character and their chromosomal status was obtained. This study was based on the findings related to the endemic nickel hyperaccumulating species on serpentine soils. However, further observations on some species and populations of *Alyssum* in Iran that grow in non-serpentine soils showed that this character does not necessarily have a complete correlation with endemism in serpentine soils (Ghasemi and Ghaderian, 2009). Some populations of *A. bractatum* from central Iran, e.g., Semirom, show nickel hyperaccumulation while growing in non-serpentine soils (not published data). Serpentines in Iran are located at northwestern, western, central, southeastern, and northeastern, but nickel hyperaccumulators have just been reported from western and north-western serpentines (Ghaderian *et al.,* 2007). Possibly, the desert climate prevents the establishment of hyperaccumulator species in other serpentine soils.

Materials and methods

Preparation of seeds: In this study, samples from different accessions of the genus *Alyssum* from different regions of Iran (Marivan, Baneh, Harsin, Meshkin Shahr, Semirom and Khalkhal; Figure 1) were collected, and their karyotypes were determined. *A. inflatum* (Marivan), *Alyssum* sp. (Baneh) and *A. bracteatum* (Harsin) are endemic to serpentine soils of western Iran. *A. bracteatum* was also collected from the populations at Semirom and Meshkin Shahr from non-serpentine soils. *A. homalocarpum*, was also used as an annual plant with seeds that are used in traditional medicine. *A. montanum*, *A. saxatile* and *Aurinia saxatilis* are cultivated as ornamental plants. *A. saxatilis* and *Alyssum saxatile* are considered synonyms in scientific names for the same species, but we examined both as two different accessions.

Seeds of the *Alyssum* sp. (Baneh), *A. inflatum* (Marivan), *A. bracteatum* (Harsin, Semirom and Meshkin Shahr) and *Alyssum* sp. (Khalkhal) were harvested from their natural habitats, where shown in figure 1. Seeds of each species/population were collected from at least 10 plants and mixed as seed pools. Seeds of *A. montanum* (Berggold) and *A. saxatile* were bought from Jelitto Company (Postfach 1264, D-29685 Schwarmstedt, Germany). *Aurinia saxatilis* seeds were prepared from Carl Pabst Company (Hauptstraße 20, 14979 Großbeeren, Germany).

Hyperaccumulation determination, catalase activity, and pigment measurement: The seeds of each plant were surface sterilized using 2.5% sodium hypochlorite and then rinsed and planted on perlite and then transferred to sterile Hoagland's solution for germination to reach the growth of at least ten leaves in the greenhouse environment with an average daily temperature of 25℃ and a night temperature of 18℃. The nutrient solution was changed every 4 days, and water evaporation was replaced by adding distilled water to the vessels. In order to qualitatively determine the possibility of nickel accumulation by these plants, all plants were treated with a concentration of 75 µM nickel in the nutrient solution, using $NiSO_4$, which was added to the nutrient solution every 2 days. Nickel accumulation was determined in the shoots after one week using dimethylglyoxime reagent, which turns pink in the presence of nickel. For this purpose, a drop of plant leaf extract was placed on a paper dipped in dimethylglyoxime. The color change indicated the characteristic of hyperaccumulation in plants (Ghasemi *et al*., 2009).

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Figure 1. Locations of 1, *Alyssum* sp. (Baneh, serpentine) at N 36.0137 and E 45.5823; 2, *A. inflatum* (Marivan, serpentine) at N 35.2290 and E 46.4623; 3, *A. bracteatum* (Harsin, serpentine) at N 34.2709 and E 47.5134; 4, *A. bracteatum* (Semirom, non-serpentine) at N 31.6482 and E 51.7325; 5, *A. bracteatum* (Meshkin Shahr, non-serpentine) at N 38.3589 and N 47.6817; and 6, *Alyssum* sp. (Khalkhal, non-serpentine) at N 37.6030 and E 48.5134.

Measurement of catalase activity in mature leaves was achieved with the method developed by Aebi (1983), and concentrations of photosynthetic pigments, including chlorophyll a, chlorophyll b and carotenoids were determined based on Lichtenthaler and Buschmann, 2001. Total extractable protein was measured by quantitative protein-dye (coomassie brilliant blue G-250) binding method (Bradford, 1976). The concentration of nickel in the shoot was determined based on acid and oxidant (H_2O_2) digestion and measurement by AAS (atomic absorption spectrophotometry, AAS-6200, Shimadzu Corporation, Chiyoda-ku, Tokyo, Japan) (Reeves *et al*., 1999).

Cultivation of seedlings in order to prepare karyotype: The seeds (n > 30) were placed in the growth chamber at a temperature of 24℃ on Hoagland's solution on the surface of a stainless steel grid. After the growth of the roots, in order to prepare suitable cells undergoing mitosis, the roots apexes were separated for a length of half to one centimeter and pretreated in the stopping mitosis solution for 2.5 hours at 4℃.

Metaphase chromosomes measurements: Stopping the division of mitosis at the metaphase stage, and staining method Alpha-bromonaphthalene was used as a pretreatment to stop mitosis in the metaphase stage, and Levitsky's solution was used for stabilization. The roots were kept in this solution for 16 hours in the refrigerator and then washed with distilled water. To prevent damage, the samples were stored in 70% ethyl alcohol at 4℃ (Olin- Fatih, 1994). Samples were stained with lacto propionic orcein for 5 hours. After washing and squashing with 45% acetic acid solution, the samples were detected with a magnification of *1000 (Agayev, 1998).

A monitoring system was used to take pictures of the karyotypes, and the images were transferred to the monitor through a Nikon video camera installed on an optical microscope. In order to measure the size of the chromosomes, the karyotype images were transferred to the software environment Micro Measure version 3.3 as a Bitmap file (Brown *et al.*, 1999), and their karyotypic formula was also determined by Levan's method (Levan *et al.*, 1964).

Karyotype analysis: The characters studied are the length of the longest chromosome (L), the length of the shortest chromosome (S), ratio of length of the longest chromosome to the shortest chromosome (L/S), intrachromosomal asymmetry (A1 = 1 – Mean S/L) and interchromosal asymmetry (A2 = standard deviation / mean of chromosomes lengths) indexes, percentage of total karyotype form (TF% = Total sum of short arm lengths/ Total sum of chromosome lengths × 100) (Huziwara, 1962), centromeric index (CI = short arm ratio to the total chromosome length), coefficient of variation of the centromeric index (average centromeric index/ standard deviation, CVci), coefficient of variation of the chromosome length (average chromosome length/ standard deviation, CVcI), chromosome asymmetry coefficient (AI= CVcI× CVci), difference in the relative length of the longest and shortest chromosome (DRL) in terms of percentage, long to short arm ratio (r-Value), and length of shortest chromosome to length of longest chromosome in percent (S%) (Paszko, 2006; Romero Zarco, 1986)

Statistical analysis: The results of karyotype measurements were the mean of at least 5 observations from 5 plants (n≥5) for each accession. Shoot nickel concentration was the mean of 3 replicates, each replicate containing 5 plants (there was 1 plant per replicate for *A. lesbiacum* because of limitation in available seed). Multiple comparisons were carried out by Duncan's test or t-test if appropriate using SPSS software version 16. Characters were analyzed into

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principal components, and the most correlated chromosomal features with the first and second components were determined.

Results

Determination of hyperaccumulator plants: Results from using DMG and nickel measurement by AAS showed that *A. inflatum*, *Alyssum* sp. (Baneh), *A. bracteatum* (Harsin, Semirom and Meshkin Shahr), and *A. lesbiacum* were able to accumulate more than 1000 $\mu g g^{-1}$DW of nickel in the shoot. Other species, i.e., *A. homalocarpum*, *A. montanum*, *A. saxatile*, *Alyssum sp.* Khalkhal) and *Aurinia saxatilis* were non-accumulators (Table 1).

Nickel effects on catalase activity, chlorophyll, and carotenoid contents: In terms of catalase activity, the plants were categorized into two groups. Results show that the activity was significantly higher in nickel hyperaccumulator plants (Table 2), among which the most activity was concerned with *Alyssum* sp. from Baneh. At exposure to 75 µM Ni, a decreased activity was observed in just non-accumulating plants, while in nickel accumulators there was no significant difference between control and treated plants. An exception was *A. bracteatum* (Semirom) which is a nickel hyperaccumulator from non-serpentine soil.

Total chlorophyll content of all accessions was changed after treatment with Ni. By treating plants with 75 µM Ni, chlorophyll content in hyperaccumulator plants was increased while in non-accumulators it was decreased (Table 2). There was no change in carotenoids content in non of the plants treated with Ni.

Chromosome number and types in *Alyssum* accessions: The results showed that all the examined *Alyssum* accessions had chromosomal numbers of 2n=16, 20 and 22. Hyperaccumulator species *A. inflatum and A. bracteatum* (Harsin), have the lowest chromosomal number (n=8), the hyperaccumulator species *A. lesbiacum* and the non-accumulator species *A. homalocarpum* and *A. montanum* have the chromosomal number (n=10), the accumulator species *Alyssum* sp. (Baneh), *A. bracteatum* (Semirom), *A. bracteatum* (Meshkin Shahr) and the non-accumulator species *A. saxatile*, *Alyssum* sp. (Khalkhal) and *Aurinia saxatilis* have the chromosomal number (n=11) (Tables 3 and 4).

The least metacentric chromosome, was observed in the non-accumulating species *A. saxatile* and the most metacentric chromosome type, was observed in *A. inflatum* as a hyperaccumulator species. Acrocentric chromosomes were observed in none of the hyperaccumulating species. The most chromosomal diversity was observed in non-accumulator *Alyssum* species from Khalkhal and *A. saxatile* (Tables 3 and 4).

In all species, except the hyperaccumulating species *Alyssum* sp. (Baneh), and the non-accumulator *A. montanum*, telocentric chromosome was observed (Tables 3 and 4).

Comparison of different chromosomal characters: Size parameters: The longest T.L (total length) belonged to *Alyssum* sp. (Baneh) and the shortest to *A. bracteatum* (Semirom). The average of T.L was 51.1 and 46.3 for non-accumulator and accumulator plants, with no significant difference between the two groups. The longest chromosome (L) was observed in *A. homalocarpum* and the shortest one (S) in *A. bracteatum* (Semirom). There was no significant difference between non-accumulators and hyperaccumulators in L and S Characters.

Total form percentage (TF%): Among the species examined in this research, the highest total form percentage value (23) was related to the nickel hyperaccumulating species *A. inflatum* and *A. bracteatum* (Harsin) and the lowest percentage of total form (12.9) belonged to the non-accumulating species *A. saxatile* (Table 7). An increase in the numerical value of this parameter indicates the karyotype symmetry. Dividing the accessions into two groups of non-accumulators and hyperaccumulators showed an average of 15.14 for the non-accumulators and 17.7 for the hyperaccumulators with no significant difference between the two groups based on a t-test at a 5% level.

Coefficient of variation of the chromosome length: CVcI in the non-accumulator species *Alyssum saxatile* was maximum (0.45) and in the hyperaccumulator *Alyssum* sp. (Baneh) was minimum (0.23). The difference between averages of the two groups of non-accumulators and hyperaccumulators was statistically significant ($P \leq 0.05$). So accumulator accessions *A. inflatum*, *A. bracteatum* (Semirom), *Alyssum* sp.(Baneh), *A. bracteatum* (Harsin), *A. bracteatum* (Meshkin Shahr), and *A. lesbiacum* were in a separated group due to the more symmetrical karyotype and species *Alyssum* sp. (Khalkhal), *Aurinia saxatilis*, *A. homalocarpum*, *A. Saxatile* and *A. montanum*, were in the second group (Table 5).

Coefficient of variation of the centromeric index (CVci): CVci was highest and lowest in *A saxatile* and *A. bracteatum* (Meshkin Shahr) respectively. The difference between the averages of two groups (non-accumulator species vs hyperaccumulator accessions) was significant ($P \leq 0.05$).

Asymmetry index (AI): The highest AI value was observed in *A. saxatile* (2.48) and the lowest was in *A. bracteatum* accession Meshkin Shahr (0.53). Average of AI for non-accumulator

species was 1.59 and for hyperaccumulator accessions it was 0.86, which the difference was statistically significant ($P \leq 0.05$).

Intrachromosomal asymmetry: The intrachromosomal asymmetry index (A1), in the studied species, showed a range of changes from 0.3 (e.g., in *A. inflatum*) to 0.5 in *A. saxatile*. Hyperaccumulator species: *A. inflatum*, *A. bracteatum* (Semirom), *Alyssum* sp. (Baneh), and *A. bracteatum* (Harsin) species, compared to *A. lesbiacum* and *A. bracteatum* (Meshkin Shahr), had more symmetrical karyotypes. The most asymmetric karyotype belonged to the *A. saxatile*

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Table 1. DMG test and accumulation of nickel in the shoot of different *Alyssum* accessions

Accession	DMG test*	Nickel concentration in shoot (µgg^{-1}DW)*	Serpentine soil substrate
A. inflatum (Marivan) (Hy.)	+	2378±361 [a]	+
Alyssum sp. (Baneh) (Hy.)	+	3929±112 [a]	+
A. bracteatum (Harsin) (Hy.)	+	2458±125 [b]	+
A. bracteatum (Semirom) (Hy.)	+	1411±234 [c]	_
A. bracteatum (Meshkin Shahr) (Hy.)	+	1022±39 [c]	_
A. lesbiacum (Hy.)	+	3899±56 [b]	+
A. homalocarpum	_	137±11 [d]	_
A. montanum	_	175±36 [d]	_
A. saxatile	_	97±28 [d]	_
Alyssum sp. (Khalkhal)	_	366±53 [d]	_
Aurinia saxatilis	_	176±39 [d]	_

***: Plants with DMG + result and Nickel concentration in shoot more than 1000 µgg^{-1}DW were considered as Nickel hyperaccumulator. Values are means of 3 replicates ± SD, (Hy.): Hyperaccumulator plant**

Table 2. Catalse activity, total chlorophyll and carotenoids contents in different accessions of *Alyssum*.

Accession	Catalase activity (U mg^{-1} protein)		Total chlorophyll (mg g^{-1} FW)		Carotenoids (mg g^{-1} FW)	
	Control	75 µM Ni	Control	75 µM Ni	Control	75 µM Ni
A. inflatum (Marivan) (Hy.)	218±21	198±9	1.4±.06	1.7±.04*	0.54±.05	0.55±.04
Alyssum sp. (Baneh) (Hy.)	286±4	288±11	1.1±.02	1.6±.03*	0.61±.03	0.58±.01
A. bracteatum (Harsin) (Hy.)	233±11	211±21	1.3±.06	1.8±.05*	0.66±.07	0.61±.07
A. bracteatum (Semirom) (Hy.)	241±25	185±6*	1.6±.1	2.1±.04*	0.58±.07	0.66±.09
A. bracteatum (Meshkin Shahr) (Hy.)	193±6	186±4	1.6±.11	2±.1*	0.67±.03	0.67±.05
A. lesbiacum (Hy.)	239±12	241±7	1.5±.09	1.8±.1*	0.61±.01	0.55±.08
A. homalocarpum	175±3	123±6*	1.9±.09	1.5±.03*	0.77±.03	0.68±.06
A. montanum	183±5	129±5*	1.6±.08	1.5±.04*	0.59±.07	0.66±.03
A. saxatile	192±2	143±8*	1.9±.07	1.3±.01*	0.72±.01	0.71±.09
Alyssum sp. (Khalkhal)	202±9	144±3*	2.2±.13	1.4±.05*	0.78±.02	0.69±.06
Aurinia saxatilis	189±4	139±6*	1.8±.04	1.2±.06*	0.61±.01	0.70±.01

***indicates a statistically significant difference between control and Ni-treated plants by t-test (P≤0.05).**
(Hy.): Hyperaccumulator plant

Table 3. Number and types of chromosomes of the hyperaccumulator *Alyssum* spp.

Accession	Karyotype formula	Chromosome number										
		1	2	3	4	5	6	7	8	9	10	11
A. inflatum (Marivan)	*6M+m+T*	M	M	M	M	M	M	m	T	-	-	-
A. bracteatum (Harsin)	*M+4m+2sm+T*	sm	m	M	M	M	Sm	m	T	-	-	-
A. lesbiacum	*M+6m+2sm+T*	M	m	M	M	sm	M	m	m	sm	T	-
Alyssum sp. (Baneh)	*M+5m+4sm+st*	M	m	M	M	m	Sm	m	st	sm	sm	sm
A. bracteatum (Meshkin Shahr)	*5m+3sm+2st+T*	sm	m	Sm	St	m	M	sm	st	m	m	T
A. bracteatum (Semirom)	*3M+4m+2sm+st+T*	M	st	M	M	sm	M	m	M	sm	M	T

M: Absolutely metacentric chromosome, m: Almost metacentric chromosome, sm: Submetacentric chromosome, t: Acrocentric chromosome, st: Sub telocentric chromosome, T: Telocentric chromosome

Table 4. Number and types of chromosomes of the non- accumulator species

Species	Karyotype formula	Chromosome number										
		1	2	3	4	5	6	7	8	9	10	11
Aurinia saxatilis	*3M+4m+sm+t+st+T*	m	st	M	m	M	M	t	m	sm	m	T
Alyssum montanum	*7m+sm+st+t*	sm	st	m	m	m	m	m	m	m	T	-
A. homalocarpum	*M+4m+4sm+T*	sm	m	sm	m	M	m	m	sm	sm	T	-
Alyssum sp. (Khalkhal)	*2M+4m+3sm+t+T*	m	sm	m	m	sm	m	sm	M	M	t	T
A. saxatile	*2M+2m+4sm+t+st+T*	st	sm	M	M	sm	sm	m	m	t	sm	T

M: Absolutely metacentric chromosome, m: Almost metacentric chromosome, sm: Submetacentric chromosome, t: Acrocentric chromosome, st: Sub telocentric chromosome, T: Telocentric chromosome

species. *Aurinia saxatilis*, *A. homalocarpum*, *Alyssum* sp. (Khalkhal), and *A. montanum*, were more symmetrical than *A. saxatile* based on the intrachromosomal asymmetry index (Table 5). The difference between the averages of two groups (non-accumulators vs. hyperaccumulators) was significant (P≤0.05).

Interchromosomal asymmetry (A2): The highest amount of A2 index was observed in *Alyssum* sp. from Khalkhal (0.48), and the lowest amount was determined for *Alyssum* sp. from Baneh (0.23). The difference of averages of the two non-accumulator and

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Table 5. Karyotype features of the studied *Alyssum* accessions

Species	T.L	L	S	L/S	r-Value*	TF%	CVcI*
Aurinia saxatilis	52.14[a]	7.71 [a]	2.7 [a]	2.84 [a]	1.71[d]	17.2 [a]	0.33 [ac]
Alyssum homalocarpum	55.51[b]	8.74 [b]	2.61 [b]	3.34 [b]	1.65[c]	14.7 [b]	0.35 [a]
A. montanum	45.96[c]	6.37[c]	2.47 [c]	2.58 [c]	1.55[c]	15.8 [c]	0.30 [ca]
Alyssum sp. (Khalkhal)	52.8 [d]	8.42 [de]	2.15 [d]	3.9 [d]	1.44[b]	15.1 [d]	0.28 [bc]
A. saxatile	49.08 [e]	8.48 [d]	2.16 [d]	4.09 [e]	1.83[d]	12.9 [e]	0.45 [d]
A. bracteatum (Harsin) (Hy.)	46.17 [f]	8.35 [e]	3.49 [e]	2.39 [f]	1.27[a]	18.8 [f]	0.25 [b]
A. inflatum (Marivan) (Hy.)	40.41 [g]	7.01 [f]	2.59 [b]	2.7 [g]	1.23[a]	23 [g]	0.28 [bc]
A. bracteatum (Meshkin Shahr) (Hy.)	40.99 [h]	5.32 [g]	2 [f]	2.66 [g]	1.67[cd]	15.6 [c]	0.25 [bc]
A. bracteatum (Semirom) (Hy.)	37.76 [i]	5.35 [h]	1.73 [g]	3.08 [h]	1.43[b]	14.7 [b]	0.29 [bc]
Alyssum sp. (Baneh) (Hy.)	64.86 [j]	8.61 [i]	3.63 [h]	2.36 [f]	1.47[b]	14.8 [b]	0.23 [b]
A. lesbiacum (Hy.)	47.7 [k]	6.97 [j]	2.32 [i]	3.00 [i]	1.32[a]	13.3 [h]	0.29 [bc]

Common letters in each column indicate a non-significant difference (P≤0.05) between accessions according to Duncan's test.
(Hy.): Hyperaccumulator plant
*** Significant difference between averages of two groups of non-accumulator and hyperaccumulator accessions based on t-test (P≤0.05).**
T.L (µm): Total length of chromosomes; L (µm): Length of longest chromosome; S (µm): Length of shortest chromosome; L/S: Relation of length of longest chromosome to length of shortest chromosome; r-Value: Ratio of long arm to short arm; TF%: Total form percentage; CVcI: Average of chromosome length/standard deviation; CVci: Average of centromeric index/standard deviation; AI: Asymmetry index (CVcI*CVci); A1: Intrachromosomal asymmetry index; A2: Interchromosomal asymmetry index; S%: Relative length of shortest chromosome; DRL%: Difference of relative length; CI: Centromeric index.

Continue of Table 5.

Species	CVci*	AI*	A1	A2*	S%	DRL%	CI*
Aurinia saxatilis	4.02 [a]	1.34 [a]	0.4 [a]	0.33 [abc]	5.17 [a]	9.61[b]	0.39 [abc]
Alyssum homalocarpum	4.66 [b]	1.64 [b]	0.4 [a]	0.30 [ab]	4.7 [b]	11.04[c]	0.36 [ab]
A. montanum	3.64 [e]	1.11 [a]	0.4 [a]	0.30 [abd]	5.37 [c]	8.48[a]	0.38 [ab]
Alyssum sp. (Khalkhal)	4.86 [c]	1.37 [a]	0.3 [c]	0.48 [bc]	3.88 [d]	11.87[c]	0.37 [ab]
A. saxatile	5.50 [d]	2.48 [c]	0.5 [b]	0.37 [c]	5.27 [e]	12.88[d]	0.33 [a]
A. bracteatum (Harsin) (Hy.)	3.62[e]	0.93 [de]	0.3 [c]	0.25 [de]	7.55 [f]	10.53[bc]	0.4 [bc]
A. inflatum (Marivan) (Hy.)	3.28 [g]	1.14 [a]	0.3 [c]	0.28 [de]	6.43 [g]	10.94[bc]	0.43 [c]
A. bracteatum (Meshkin Shahr) (Hy.)	2.08 [h]	0.53 [e]	0.4 [a]	0.25 [de]	4.87 [h]	8.10[a]	0.37 [ab]
A. bracteatum (Semirom) (Hy.)	2.24 [i]	0.67 [de]	0.3 [c]	0.29 [abd]	4.58 [i]	9.59[b]	0.40 [bc]
Alyssum sp. (Baneh) (Hy.)	3.51 [f]	0.83 [d]	0.3 [c]	0.23 [e]	5.59 [j]	7.68[a]	0.39 [abc]
A. lesbiacum (Hy.)	3.55 [f]	1.06 [a]	0.4 [a]	0.30 [abc]	4.86 [h]	9.75[b]	0.4 [bc]

Common letters in each column indicate a non-significant difference (P≤0.05) between accessions according to Duncan's test.
(Hy.): Hyperaccumulator plant
*** Significant difference between averages of two groups of non-accumulator and hyperaccumulator accessions based on t-test (P≤0.05).**
T.L (µm): Total length of chromosomes; L (µm): Length of longest chromosome; S (µm): Length of shortest chromosome; L/S: Relation of length of longest chromosome to length of shortest chromosome; r-Value: Ratio of long arm to short arm; TF%: Total form percentage; CVcI: Average of chromosome length/standard deviation; CVci: Average of centromeric index/standard deviation; AI: Asymmetry index (CVcI*CVci); A1: Intrachromosomal asymmetry index; A2: Interchromosomal asymmetry index; S%: Relative length of shortest chromosome; DRL%: Difference of relative length; CI: Centromeric index.

hyperaccumulator plant groups was statistically significant (P≤0.05).

Relative length of shortest chromosome: The rate of changes in this parameter (S%), in species *A. saxatile* and *Alyssum* sp. (Khalkhal), was the highest amount, and in *A. bracteatum* (Meshkin Shahr), and *A. bracteatum* (Semirom), was the lowest amount (Table 5).

Difference of relative length (DRL%): The least DRL% value was calculated for *Alyssum* sp. (Baneh) and the highest value for *A. saxatile*. The average DRL% was 10.78 and 9.21 for non-accumulator and hyperaccumulator plants, respectively. The difference between these two groups was not statistically significant (P≤0.05).

Centromeric index (CI): The greatest CI value was observed for *A. inflatum* (Marivan) versus the lowest value for *A. saxatile*. The difference in averages of two groups (non-accumulator species and hyperaccumulator accessions) was statistically significant (P≤0.05).

The ratio of long arm to short arm (r-Value): There was a significant difference between non-accumulator and hyperaccumulator plants (P≤0.05). The average of r-Value was 1.64 and 1.40 for non-accumulators and hyperaccumulators respectively. The lowest values observed in *A. inflatum* (Marivan) and *A. bracteatum*

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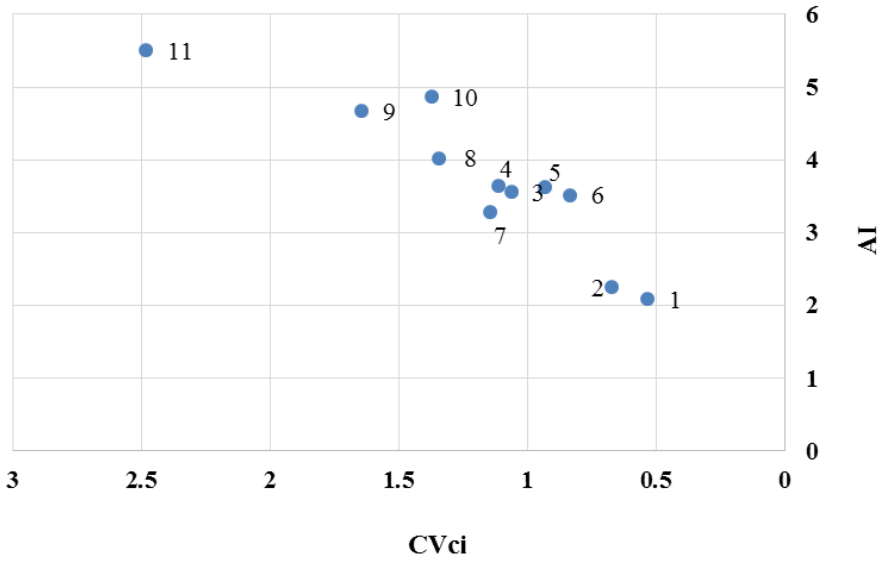

Figure 2. Diagram of the distribution of accessions based on two parameters, AI (asymmetry index) and CVci (average of centromeric index/standard deviation)
1: *A. bracteatum* (Meshkin Shahr), 2: *A. bracteatum* (Semirom), 3: *A. lesbiacum*, 4: *A. montanum*, 5: *A. bracacteatum* (Harsin), 6: *Alyssum* sp. (Baneh), 7: *A. inflatum* (Marivan), 8: *Aurinia saxatilis*, 9: *A. homalocarpum*, 10: *Alyssum* sp. (Khalkhal), 11: *A. saxatile*

(Harsin), and the highest values in *A. saxatile* and *Aurinia saxatilis*.

Analysis of karyotypic features into principal components and examining accessions distributions: Analysis into principal components based on the average of 14 characters showed that the first extracted component was most of all related to the asymmetry index (AI), the second most correlated parameter was the CVci, and the third one was the CVcI.

The studied accessions were divided into 4 different groups by examining the distribution in correlation with AI and CVci (Figure 2). *A. saxatile*, with the most distance, compared to other species, was placed in a separate group. *A. lesbiacum, A. bracacteatum* (Harsin), *Alyssum* sp. (Baneh), and *A. inflatum* (Marivan), were placed in one group, *Aurinia saxatilis, Alyssum* sp. (Khalkhal), *A. montanum*, and *A. homalocarpum* were placed in the third group, and *A. bracteatum* (Meshkin Shahr), and *A. bracteatum* (Semirom), were arranged in the fourth category.

Discussion

The two main findings of this research are (a) hyperaccumulator accessions in comparison to the non-accumulator species have more symmetric karyotypes, and (b) non-serpentine accessions of *A. bracteatum* show more asymmetric karyotypes and more chromosome numbers in comparison to the serpentine accession with decreased nickel hyperaccumulation ability in non-serpentine accessions. Significant differences between non-accumulators and hyperaccumulators were observed in parameters including r-Value, AI, CI, CVci and CVcI as indicators of asymmetry and more evolved karyotypes. Categorization shown in figures 2 and 3 also divides these two groups from each other. Another indicator feature that appears in karyotype evolution is a secondary constriction in the chromosomes and the presence of satellites. This feature was observed in *A. saxatile* in chromosome number 1, and the reason for this could be the heritability of hyperactivity of the nucleolar organizing regions (NORS) (Armstrong *et al*., 2001) and the activity of rDNA genes (Olin-Fatih, 1994; Maluszynska and Heslope-Harrison, 1993).

Cytotaxonomic studies on some *Alyssum* species showed that the chromosome number and karyotype in *A. minus*, *A. foliosum*, and *A. strigosum* species, is 2n=16, and in *A. alyssoides* is 2n=32 (Dudley, 1965). The basic chromosome number for the three studied species of *Alyssum* in Iran, was n=8 (Warwick and Al-Shehbaz, 2006). Sirin *et al*. (2020) reported 2n=16 in some *Alyssum* species from Turkey, and the basic chromosome number for the *Alysseae* tribe is 8 (Warwick *et al.*, 2008). The chromosome number n=8 was observed in *A. bracteatum* (Harsin) and *A. inflatum* (Marivan) as serpentine nickel hyperaccumulator plants. The difference in the chromosome number of studied diploid species, which had basic chromosome numbers 8, 10 and 11, and karyotypic variations could be as a result of some of the reasons for diversification and speciation in these species. Anatolia is considered a center for *Alyssum* formation and diversity. However, extension to the east through Iran's plateau could increase its diversity, evolution, and fitness to new habitats.

Karyotypic analyses of some wild species belonging to the genus *Brassica* (from a cytological point of view), showed that, species with a higher number of metacentric chromosomes and less chromosomal diversity, had symmetrical karyotypes (Fahleson *et al.*,

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1994). In the symmetric karyotype, the chromosomes are almost the same size and metacentric, and this type of karyotype is more primitive in terms of evolution, and in the asymmetric karyotype, the percentage of TF and centromeric index is low (Luo *et al*., 2003), and chromosomes are submetacentric, or most of them are acrocentric and telocentric (Rohami *et al.*, 2010).

Comparing chromosome types showed that in *A. inflatum* (Marivan), 7 out of 8 chromosomes are metacentric (absolutely metacentric and almost metacentric), while it is 4 out of 11 in *A. saxatile*. The average of the ratio of metacentric to total chromosomes in serpentine endemic and non-serpentine plants was 0.71 and 0.55, respectively. Although the difference between the two groups was not significant (P=0.15), this ratio was 0.54 in the two accessions of non-serpentine hyperaccumulators (*A. bracteatum*, accessions of Semirom and Meshkin Shahr). Observing such similarity in the non-serpentine but hyperaccumulator accessions suggests a tendency for diversification and adaptation to new habitats. In species with metacentric chromosomes, the karyotypic symmetry was primitive type and indicated the early stages of species evolution (Lysak and Weiss-Schneeweiss, 2021).

There are two hypotheses regarding the establishment of the nickel hyperaccumulation trait in plants. First, it is assumed that the trait has evolved in different taxons independently in response to the environment. Second, the trait has appeared in ancestral plants and then dispersed in different evolved lineages (Sobczyk *et al*., 2017; Pollard *et al*., 2002). The results of this research are well-matched to the second hypothesis since the most primitive characters are observed in the serpentine endemic nickel hyperaccumulators. In addition, non-serpentine nickel hyperaccumulators of *A. bracteatum* (Semirom and Meshkin Shahr) showed more advanced traits in comparison to their relative serpentine accession *A. bracteatum* (Harsin). In other words, we observed a direct correlation between hyperaccumulation and primitive chromosomal traits. This suggests that the nickel hyperaccumulation trait evolved when the taxon was in a primitive state.

Sobczyk *et al*. (2017), by studying the polymorphism of two putative genes responsible for fitness to ecological conditions of serpentine soils and comparing them to the polymorphism of a non-relevant gene in serpentine appropriateness, showed that serpentine and non-serpentine populations of *Alyssum serpyllifolium* did not highly differ from each other and higher tolerance to nickel in serpentine populations could be due to local adaptations to serpentine soils. Our results showed that non-serpentine accessions of *A. bracteatum* (Semirom and Meshkin Shahr) were categorized into different groups from its serpentine accession (Harsin). Although there is no report to show whether they all belong to the same species or a speciation has occurred, it could be concluded that some changes have occurred to adapt them to the non-serpentine substrates.

The inadvertent uptake hypothesis (IUH) could be another explanation for the evolution of heavy metal accumulation by plants due to colonization in nutrient-deficient substrates (Meindl *et al*., 2021; Cappa and Pillon-Smith, 2014), so plants with high efficiency in taking up the nutrients can survive in these environments (e.g., serpentine soils). In addition to the nutrient elements, they inevitably would take up heavy metals, and they have to detoxify them in different tissues. However, if they disperse to other normal substrates and suitable climates to survive, they no longer need such an extended uptake system. So metal hyperaccumulator plants could ease or decrease the ability of metal accumulation. Another result could be that the direction of evolution is from serpentine soil toward non-serpentine soils. At least a highly efficient calcium uptake system has been suggested in serpentine plants since the ratio of Ca/Mg is very low in these soils (Ghasemi *et al.*, 2015; Ghasemi and Ghaderian, 2009; Asemaneh *et al.*, 2007), and evidence for interference of Ca and nickel tolerance has been shown in *A. inflatum* (Ghasemi *et al*., 2018). However, increased activity of uptake systems could overload minerals and induce lethal reactions, as shown in cell suspension culture of *A. inflatum* in response to higher calcium concentrations in medium (Ghasemi *et al*., 2020). In this study we observed diminished ability of nickel accumulation by two non-serpentine accessions of *A. bracteatum* (Semirom and Meshkin Shahr) in comparison to the serpentine accession (Harsin). So possibly they have partially lost their prolonged element uptake system after adaptation to non-serpentine soils. In addition, there is a definition for obligate and facultative hyperaccumulator plants, which grow on metalliferous or non-metalliferous soils (without exhibiting metal hyperaccumulation), respectively (Pollard *et al*., 2014). Based on this definition, *A. bracteatum*, *A. inflatum* (based on other reports, Ghasemi and Ghaderian, 2009), and other serpentine endemic hyperaccumulators such as *Alyssum* sp. (Baneh) and *A. lesbiacum* could be categorized as obligate hyperaccumulators.

In this research we studied an accession of *Alyssum* from Khalkhal. It has high morphological similarity to *A. homalocarpum*, and its chromosomal feature indicates that they must be closed species with more advanced characters. Clustering also showed that *Alyssum* sp. (Khalkhal) was placed in the group including *A. homalocarpum*. *Alyssum* sp. (Baneh) is also similar to *A. inflatum* (Marivan) in terms of morphology; both are nickel hyperaccumulators and serpentine endemics, but they showed differences, including higher capacity of nickel accumulation in *Alyssum* sp. (Baneh), and chromosomal characteristics. Indeed, they were not considered as the same species, and more studies are needed to determine if they are either different species or variations of the *A. inflatum*.

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Conclusion and outlooks

Nickel hyperaccumulator *Alyssum inflatum* was the species with the most primitive chromosomal features among the studied accessions, while *A. saxatile*, which can't accumulate nickel, has advanced chromosomal characters. We suggest that nickel hyperaccumulation has evolved in limited serpentine endemic species, expanded through speciation and colonization on different serpentine (and non-serpentine) substrates, and could be removed after adaptation to normal soils. Based on this, more studies are needed to determine whether further evolution and chromosomal changes in non-accumulating plants, have caused the elimination of the Ni-accumulation character or whether the hyperaccumulation character in these plants, has emerged independently and without a clear connection with the chromosomal status, and it is just a species adaptation to their environments. Finding specific and directly responsible genes in nickel hyperaccumulation, and determining their evolutionary changes in different accessions of species such as *A. bracteatum* or *A. inflatum* will help to find the origin of metal hyperaccumulation. It is also important to determine types of chromosomal rearrangements as driving forces in the evolution of nickel hyperaccumulation and adaptation to serpentine soils.

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