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Development and diversity analysis of an hexaploid pre-breeding asparagus population with introgressions from wild relative species

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ABSTRACT

The development of new asparagus (*Asparagus officinalis* L.) cultivars has been hindered by the lack of genetic diversity in the crop. The current study aims at generating novel plant material to widen the genetic base of the crop by using CWR (crop wild relatives). We have developed a hexaploid population of about 1000 plants with introgressions of four polyploid CWR species (*A. maritimus, A. pseudoscaber, A. brachyphyllus* and *A. macrorrhizus*). The population is the second generation from a first backcrossing using the landrace 'Morado de Huétor' as recurrent parent and was obtained in open pollination. The diversity of the population was analyzed by six EST-SSR (Expressed Sequence Tag-Simple Sequence Repeat) markers. A random sampling of 60 plants was used to estimate the genetic variability. The results were compared with data previously obtained from the parental collection, the landrace 'Morado de Huétor' and diploid current asparagus cultivars. The average PIC_m (polymorphic information content) value of the new population and 'Morado de Huétor' were similar (>0.8) and higher than the value observed in the diploid cultivars (0.61). At least 22.2% of the alleles detected in the hexaploid population were specific from the CWR species used in this study. Principal coordinate analyses (PCoA) revealed a clear genetic diversity differentiation between populations. The population of 1000 plants was evaluated for agronomic traits (earliness, stalk number, branching height and stalk thickness) for two years and we selected 80 plants to develop a breeding base population. Variation was significant for all traits ($P<0.001$). These 80 plants were also analyzed with the six EST-SSR markers and they conserve the variability present in the starting population. The results show an increase in the genetic variability and offer a new opportunity for asparagus improvement.

1. Introduction

Garden asparagus (*Asparagus officinalis* L., 2n =2x = 20) is an herbaceous, perennial and dioecious species belonging to the *Asparagaceae* family. *Asparagus* is a large genus that includes more than 200 species distributed in temperate regions of Africa, Asia, Australia and Europe (Kubota et al., 2012; "The Plant List.," 2013) . Taxonomically, the genus is divided into three subgenera including dioecious (*Asparagus* subgenus) and hermaphroditic (*Myrsiphyllum* and *Protasparagus* subgenera) species (Clifford and Conran, 1987). A broad variation of the ploidy level (2x, 4x, 6x, 8x, 10x and 12x) has been reported for the *Asparagus* genus, suggesting that polyploidization may have played an important role in the evolution of this genus (Castro et al., 2013).

Garden asparagus is the economically most important *Asparagus* species cultivated worldwide as a vegetable crop. In 2018, the area harvested reached 1584,544 ha, a figure similar to other crops such as garlic, green beans and eggplant (FAO, 2021). Nearly all asparagus production in the world comes from diploid cultivars. Most of these cultivars derive from the Dutch population 'Violet Dutch' (Knaflewski, 1996) and, as a consequence, they have a narrow genetic base (Geoffriau et al., 1992; Mercati et al., 2015; Moreno et al., 2006). The resulting genetic base presents difficulties for asparagus breeders now seeking to develop new cultivars. With the anticipated climate change, there is a rising demand to develop improved asparagus varieties with a higher yield, and pest and disease resistant to meet the needs of the burgeoning population throughout the world. So, it is vitally important to have access to a greater gene pool than the currently available (McCouch et al., 2013). The crop wild relatives (CWR) offer an opportunity to broaden the genetic base of the crops (Dempewolf et al., 2014; McCouch et al., 2013). However, much of the genetic diversity available

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in CWR remains largely unexploited for breeding (Prohens et al., 2017). In asparagus, there is a group of *A. officinalis* wild relatives distributed across different regions of Europe (*A. prostratus* Dumort., *A. maritimus* (L.) Mill., *A. pseudoscaber* Grecescu, *A. brachyphyllus* Turcz., *A. kasakstanicus* Iljin, *A. tenuifolius* Lam., *A. macrorrhizus* Pedrol, Regalado et López-Encina) and Asia (*A. persicus* Baker, *A. breslerianus* Schult. & Schult. f. and *A. verticillatus* L, *A. kiusianus* Makino, *A. oligoclonos* Maxim) that could be used for crop improvement (Komarov et al., 1935; Kubota et al., 2012; Valdes, 1980; Xinqi and Tamanian, 2000). Wild relative species in the genus *Asparagus* are sources of variation for many agricultural traits such as salt tolerance, drought tolerance, soil acidity tolerance, and disease resistance that are important for the crop (Kanno and Yokoyama, 2011; Nothnagel et al., 2014; Venezia et al., 1993). In addition, some wild species are adapted to stressful environments and have characteristics that the cultivated species does not have. For instance, there are *A. macrorrhizus* populations located very close to the marine coast and *A. breslerianus* populations can grow in a hot and dry climate (Mousavizadeh et al., 2015; Regalado et al., 2017). Also, CWR could be used in the genetic improvement of this vegetable as a functional food. Higher content in saponins, a family of bioactive compounds, has been reported in a set of CWR species when compared to *A. officinalis* (Jaramillo-Carmona et al., 2017).

Because all the above mentioned, it seems perfectly reasonable to use CWR for developing new cultivars and broadening the asparagus genetic base. A group of asparagus wild relatives including A. *prostratus* (4x), A. *pseudoscaber* (6x), A. *brachyphyllus* (4x, 6x), *A. maritimus* (4x, 6x), A. *macrorrhizus* (12x) are polyploid. Also, different wild polyploid populations (4x, 8x, 10x) have been found in *A. officinalis* (Mousavizadeh et al., 2016). In cultivated asparagus, some tetraploid landraces are cultivated on a local scale. 'Violetto d'Albenga' and 'Morado de Huétor' are grown in Italy and Spain, respectively whereas other two landraces ('Cereseto' and 'Poire') are grown in Argentina (Falavigna and Fantino, 1985; López Anido et al., 2000; Moreno et al., 2006). The landraces cultivated in Europe could have evolved from natural interspecific origin between *A. officinalis* and *A. maritimus* (Moreno et al., 2008; Riccardi et al., 2011). Some tetraploid cultivars have been obtained from 'Violetto d'Albenga' ('Purple Passion', 'Dulce Verde', 'Purple Pacific', 'NJ1016' and 'Sweet Purple') and an octoploid cultivar ('HT801') was obtained from 'Morado de Huétor' (Benson et al., 1996; Falloon and Andersen, 1999; Moreno et al., 2012; Wehner, 2002). Some studies employing the landrace 'Morado de Huétor' and wild asparagus populations revealed a higher genetic variability compared to current cultivars (Castro et al., 2014, 2013; Moreno et al., 2006; Mousavizadeh et al., 2018). Taken together, all these studies suggest that asparagus landraces and wild polyploid species may be a source of new alleles or genes potentially useful for breeding asparagus for yield, nutritional quality, adaptability, and resistance to different biotic and abiotic stresses affecting the crop.

The development of new germplasm should be a priority in asparagus breeding programs. Despite the importance of this goal in asparagus breeding, there are very few studies focused on the development of new pre-breeding germplasm. The most comprehensive studies have been carried out employing tetraploid accessions. In this sense, a collection of diploid plants carrying germplasm from two landraces ('Morado de Huétor' and 'Violetto d'Albenga') and two wild species (*A. maritimus* and *A. acutifolius* L.) were obtained (Castro et al., 2014; Regalado et al., 2016; Riccardi et al., 2011). Moreover, a set of polyploid plants (4x, 6x) carrying germplasm from 'Morado de Huétor' and different wild relative polyploid species including 4x (*A. prostratus*), 6x (*A. maritimus, A. pseudoscaber, A. brachyphyllus*) and 12x (*A. macrorrhizus*) were recently obtained (Amian et al., 2018).

The development of diploid and polyploid breeding base populations with a wide genetic variability carrying introgressions from different wild species may facilitate obtaining new parents which can be used to broaden the genetic base of garden asparagus..

In this study, we developed a hexaploid pre-breeding population with introgressions of germplasm from CWR, and we analyzed its genetic diversity by EST-SSR (Expressed Sequence Tag-simple Sequence Repeat) markers.

2. Materials and methods

2.1. Plant material

A collection of 46 hexaploid plants previously developed by Amian et al. (2018) was used to develop an hexaploid population of 1000 individuals (Fig. 1). *Asparagus maritimus* (6x), *A. pseudoscaber* (6x) *A. brachyphyllus* (6x) and *A. macrorrhizus* (12x) were used as CWR species in the collection developed by Amian et al. (2018) . The 46 plants were crossed under open pollination. The ploidy level of these plants was confirmed by flow cytometry methodology described in Moreno et al. (2008). Seeds from all female plants ($n = 20$) were harvested in autumn 2015. A balanced mix of seeds of each female plant was grown in a seedbed in the greenhouse. In the spring 2016, the seedlings ($n = 1000$) were transplanted in an experimental field at the research center of the regional government of Andalusia (IFAPA), Córdoba, Spain. The distances between plants and rows in the field plot were 0.5 m and 1.5 m, respectively. The standard cultural practices were applied for growing the plants.

2.2. SSR marker analysis

A sample of 60 individuals was randomly chosen to assess the genetic variability of the population. Total genomic DNA of each individual was isolated from 1 g of tips from young spears using a modified CTAB method (Torres et al., 1993). DNA was quantified based on absorbance at 260 nm with the NanoDrop ND-1000 (Thermo SCIENTIFIC, Waltham, MA). Six EST-SSR markers (AG7, AG8, TC1, TC3, TC7 and TC9) developed in diploid current cultivars by Caruso et al., 2008 were used to characterize the individuals. These SSR markers amplified in tetraploid landrace 'Morado de Huétor' and wild species (Castro et al., 2013; Moreno et al., 2010a). Forward primers were synthesized with fluorescent dyes 6FAM or HEX (Applied Biosystems) at the 5′ ends. These markers were selected using the following criteria: i) the polymorphic information content (PIC) value was high (> 0.8) according to previous studies using the landrace 'Morado de Huétor' (Moreno et al., 2010a) and ii) amplification of a clear and reproducible peak pattern in all individuals. The markers are independent and distributed along 4 chromosomes (V, VI, VII and X) out of the 10 basic chromosome number of this species. It should be noted that even though the markers AG7 and TC1 (on chromosome V) and AG8 and TC9 (on chromosome VII) are on the same chromosome, the genetic distance between them in a saturated genetic map of *A. officinalis* (Moreno et al., 2018) was high (> 120 cM), indicating that they are distributed in different regions of the asparagus genome.

Amplification of the markers was performed as in Caruso et al., 2008. The PCR products were separated using an automated capillary sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems/HITACHI, Madrid, Spain). The size of the amplified bands was calculated based on an internal DNA standard (400HD-ROX) with GeneScan software (version 3.x) and the results were interpreted using the Genotyper program (version 3.7) all from Applied Biosystems. Due to the polyploid nature of the new population and the difficulty to infer allele frequency, SSR marker alleles were scored as presence (1) or absence (0) of band for each accession and a binary data matrix was created. The PIC value of each marker was calculated using the formula $PIC_m = 1 - \sum Pj^2$, where Pj is the band frequency of the j^{th} allele. To study the genetic diversity between populations we have employed the Principal coordinate analyses (PCoA), which has turned out to be a useful tool in comparative studies with different ploidy levels (Kloda et al., 2008). PCoA was performed by genetic distance matrix,calculated from binary data, with the distance-standardized method using GENALEX 6.5 software (Peakall

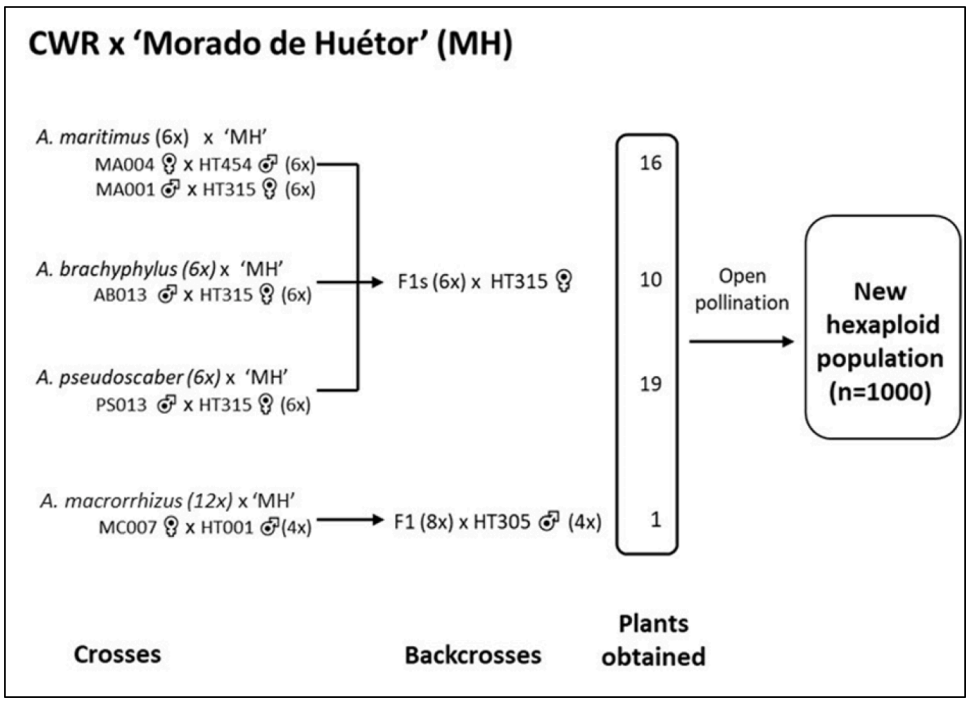

Fig. 1. Scheme of crosses and backcrosses carried out to introgress CWR germplasm into asparagus landrace 'Morado de Huétor'. AB, PS, MA, MC and HT stand for *A. brachyphyllus, A. pseudoscaber, A. maritimus, A. macrorrhizus* and the landrace 'Morado de Huetor' respectively. In brackets ploidy levels.

and Smouse, 2012).

The results of the 60 individuals were compared with the original hexaploid collection (n = 46), the tetraploid landrace 'Morado de Huétor' (n = 38) and a set of six diploid commercial cultivars (n = 63) with American and European origin that were also analyzed with these six EST-SSR markers. In order to compare the diploid and the polyploid population, the alleles in the diploids were also scored as presence (1) or absence (0). It should be mentioned that the original hexaploid population, the tetraploid landrace and the diploid cultivars were analyzed in previous studies (Amian et al., 2018; Castro et al., 2014). However, in the current study we used some markers, which were not used in the previous studies. To measure the genetic variation observed in the biplot from PCoA, the mean value of pairwise distance was calculated in each population. The smallest the mean distance, the lowest variation. Student's t-test was employed to compare the mean values.

2.3. Assessment of morpho-agronomic traits

The hexaploid population (n = 1000) was phenotyped for five morpho-agronomic traits. The following traits were evaluated in 2016 and 2017 by the end of the vegetative season (November): stalk number per plant, scored using a 1–3 rating scale (1 indicates less than 10 stalks per plant, 2 = 10–20 stalks, and 3 indicates more than 20 stalks per plant); branching height, which is highly correlated with the tightness of spear tips (Ellison, 1986), measured as distance between soil level and first branch and evaluated following a 1–3 rating scale (1 indicates less than 15 cm distance between soil and the first branch, 2 = 15–40 cm, and 3 indicates more than 40 cm); stalk thickness, evaluated at 10 cm from soil level, scored using a 1–3 rating scale (1 = ⟨ 10 mm, 2 = 10–15 mm, and 3 = ⟩ 15 mm).

Earliness in spear production was evaluated in 2017 and 2018 in a single day at the beginning of the spring (March). The trait was evaluated by the number of spears per plant and the phenological stage reached by the spears of each plant. The phenological stage was measured using a 0–4 rating scale (0 = no spears, 1 = spears below 20 cm long, 2 = spears at least 20 cm long without primary branches, 3 = spears developing to stalks with primary branches, 4 = stalks with primary and secondary branches).

Analysis of variance for two years was applied to the data according to the following model:

$$x_{ij} = \mu + A_i + Y_j + e_{ij}$$

where x_{ij} is the individual datum, μ the general mean, A_i the effect of ith plant, Y_j the effect of jth year and e_{ij} is the residual error.

Based on the morpho-agronomic trait data, 80 plants of the population were selected and crossed between them in open pollination. Seeds from the female plants were harvested to be used in the future to develop a breeding base population. The genetic variability of these selected plants was analyzed using the six EST-SSR markers previously mentioned and compared with the samples taken at random (n = 60) from the population by PCoA.

3. Results

3.1. Genetic diversity analysis

All plants of the parental population were hexaploid according to the flow cytometry analysis. The genetic diversity of the new hexaploid population derived from the parental one was analyzed using six EST-SSR markers (Table 1). The total number of alleles per marker ranged from 6 to 19 for TC9 and TC3, respectively. PIC_m values ranged from 0.78 to 0.89 with a mean value of 0.84. All the markers were highly polymorphic and detected a total of 81 alleles. Eighteen out of the 81 alleles were identified only in the wild species, indicating that they were wild-specific alleles. Specific alleles from two different wild species were identified in 36% of the hexaploid plants analyzed, suggesting that they have germplasm introgressions from two CWR species. Most specific alleles came from the hexaploid species *A. maritimus, A. pseudoscaber* and *A. brachyphyllus* (Table 2). There was only one specific allele from the dodecaploid *A. macrorrhizus* that was also present in the remaining species. The number of plants with introgressions from *A. maritimus, A. pseudoscaber* and *A. brachyphyllus* in the parental population was higher (Fig. 1), and that may explain the highest number alleles found in the

Table 1
Number of alleles and polymorphic information content (PIC_m) observed in asparagus diploid cultivars, the tetraploid landrace 'Morado de Huétor', the hexaploid parental population and the new hexaploid population with introgressions from CWR species developed in this study.

	Diploid cultivars[a] ($n = 63$)		Tetraploid landrace[a] ($n = 38$)		Hexaploid parental population[a] ($n = 46$)		New hexaploid population[a] ($n = 60$)	
EST-SSR locus	No. Alleles	PIC_m	No. Alleles	PIC_m	No. Alleles[b]	PIC_m	No. Alleles[b]	PIC_m
AG7	3	0.521	8	0.772	10 (3)	0.762	10 (3)	0.804
AG8	4	0.488	14	0.884	15 (2)	0.846	16 (2)	0.886
TC1	3	0.607	14	0.872	15 (3)	0.825	15 (3)	0.856
TC3	5	0.739	15	0.837	19 (5)	0.885	19 (5)	0.895
TC7	6	0.716	8	0.808	14 (3)	0.802	15 (3)	0.833
TC9	4	0.620	13	0.841	6 (2)	0.746	6 (2)	0.784
Total	25		72		79 (18)		81 (18)	
Mean	4.2	0.615	12	0.836	13.2	0.811	13.5	0.843

[a] In brackets number of individuals analyzed per population.
[b] In brackets number of specific alleles from wild species.PICm : Polymorphic information content estimated using band frequency of alleles into population.

Table 2
Alleles size (bp) and number of specific alleles from CWR species present in the new hexaploid asparagus population.

EST-SSR locus	*A. maritimus*	*A. brachyphyllus*	*A. pseudoscaber*	*A. macrorrhizus*	Total
AG7	176, 183	181	181		3
AG8	230		256		2
TC1		230	214, 222		3
TC3	140, 154	165, 169	138, 169		5
TC7	239	199	192		3
TC9	155	155	144, 155	155	2
Total					18

new hexaploid population. These results suggest that these three species may have had a greater contribution in the new population. On the other hand, crossing between different ploidy levels is usually less successful, and that may explain a lower contribution of *A. macrorrhizus* in the new population. Comparisons among the hexaploid population, samples from the diploid cultivars and the tetraploid landrace 'Morado de Huétor' revealed that the total number of alleles detected in the hexaploid population (81 alleles) was higher than the number of alleles detected in the diploid cultivars (25 alleles) and the tetraploid landrace 'Morado de Huétor' (72 alleles). The mean PIC_m value of the new hexaploid population was similar to the value observed in the tetraploid landrace 'Morado de Huétor' and higher than the value in the diploid cultivars (Table 1). These results suggest that the new hexaploid population may have higher allelic diversity than the diploid cultivars and the tetraploid landrace.

Principal coordinates analyses were conducted to compare the genetic diversity between the new hexaploid population, the diploid cultivars, the tetraploid landrace 'Morado de Huétor', and the parental population (Fig. 2). It was observed that the first and second principal coordinates accounted for 65.6% of the total variation. The diploid cultivars, the tetraploid landrace, the new hexaploid and parental populations were clearly separated into three different groups, indicating that their genetic variability were different from each other. All the individuals from the diploid cultivars group were closely clustered, while the individuals from the tetraploid landrace 'Morado de Huétor' and the hexaploid populations presented a wider distribution within each group. When comparing the hexaploid populations, the individuals from the new population presented a wider distribution than the parent collection. The distance mean values between pair of individuals were significantly higher ($P < 0.001$) in the new hexaploid population than in the diploids, 'Morado de Huétor' and parental population (Table 3). The higher genetic variation of the new population compared to the parental one was probably a result of the new genetic combinations in the hexaploid population.

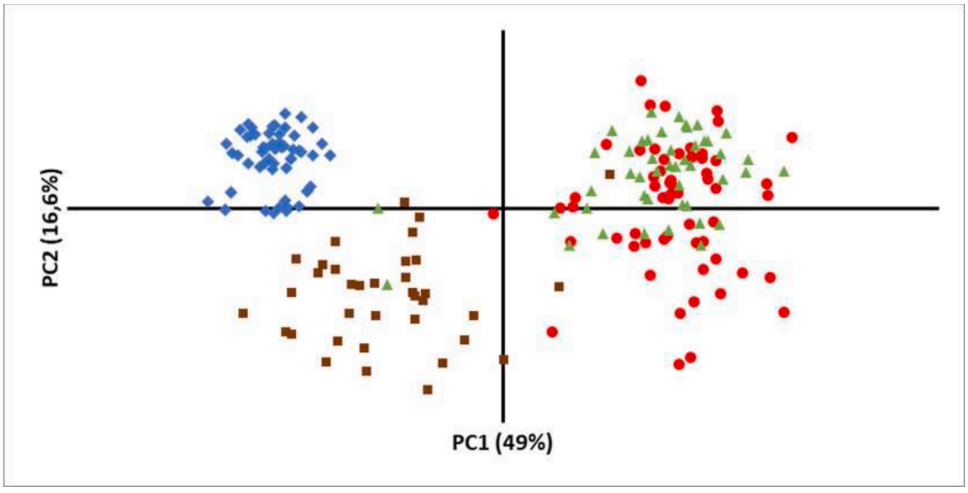

Fig. 2. PCoA analysis based on profiles from EST-SSR markers analyzed in diploid cultivar (♦), tetraploid landrace 'Morado de Huétor' (■), hexaploid parental collection (▲) and new hexaploid population selected by random sampling (●).

Table 3
Number of individuals per population and mean ± standard error (SE) of distances between pairs of individuals for different asparagus populations.

Populations	N	Mean ± SE
New hexaploid	60	20.47 ± 0.117
Parental hexaploid	46	16.18 ± 0.160***
'Morado de Huétor'	38	18.78 ± 0.157***
Diploid cultivars	63	8.31 ± 0.070***
Hexaploid selected plants	80	20.59 ± 0.092

*** Significantly different ($P < 0.001$) from the new hexaploid population (bold) by *t*-Student test.

3.2. Phenotypic evaluation

The new hexaploid population was evaluated for different morpho-agronomic traits for two years (Fig. 3). Earliness in spear production was evaluated by two different traits: number of spears per plant and phenological stage of the plants. The correlation between these traits was significant with a correlation coefficient of 0.51 and 0.60 in 2017 and 2018, respectively.

The five traits showed highly significant variation between individuals ($P<0.0001$), suggesting that there is genotypic variation in this population for all the traits (Table 4). Based on the phenotypic data, 80 plants were selected as parents to develop a breeding base population. The 80 selected parent plants showed mean values higher than the mean of the total population (Table 5). These selected plants were also genotyped with the six EST-SSR markers, and the genetic diversity was compared with that of the whole population. PCoA results revealed a similar genetic diversity between them with non-significant differences between the mean values of distance between pairs of individuals (Table 3) (Fig 4).

4. Discussion

It is well known that the current diploid cultivars of garden asparagus have a common origin, the Dutch population 'Violet Dutch' and, as a consequence, they have a narrow genetic base (Geoffriau et al., 1992; Knaflewski, 1996; Mercati et al., 2015; Moreno et al., 2006). Our results agree with previous studies and confirm the narrow genetic base of diploid cultivars. Despite the potential value of asparagus wild relative species as new sources of genes of interest, they are underutilized for developing new asparagus germplasm. In fact, the incorporation of genetic diversity from wild relatives in cultivated asparagus has lagged behind other crops (Hajjar and Hodgkin, 2007), likely in part because differences in ploidy complicate use of wild asparagus in breeding. New collections may enable breeders to address pressing needs to meet the challenges of climate change.

In this study, a hexaploid population with introgression from several CWR (*A. maritimus* (6x), *A. pseudoscaber* (6x) *A. brachyphyllus* (6x), *A. macrorrhizus* (12x)) was obtained.. The EST-SSR markers and PCoA results revealed that the genetic diversity in the hexaploid population was different and higher than the diversity detected in the current diploid cultivars. And, therefore, the hexaploid population may be useful to enlarge the genetic base of the cultivated asparagus. The 80 plants selected from the population because they had a good agronomic performance also conserve a high genetic diversity. In the future, the offsprings of these plants obtained by open pollination will form a base population for breeding.

Table 4
Analysis of variance for five traits evaluated for two years in an hexaploid population of cultivated asparagus with introgressions from wild germplasm.

Traits[b]	Mean Square[a] Year df = 1	Individuals df = 944	Error df = 940
Spear number	2632.25 (<0.0001)	43.63 (<0.0001)	13.98
Phenological stage	283.06 (<0.0001)	1.66 (<0.0001)	0.69
Stalk number	5.09 (<0.0001)	0.19 (<0.0001)	0.05
Branching height	1.76 (<0.0001)	0.06 (<0.0001)	0.02
Stalk thickness	1.05 (<0.0001)	0.07 (<0.0001)	0.02

[a] In brackets *P* value.
[b] Spear number per plant and phenological stage were evaluated following a 0–4 rating scale (0 = no spears, 1 = spears < 20 cm, 2 = spears >20 cm without primary branches, 3 = spears with primary branches and 4 = spears with secondary branches); Stalk number, branching height and stalk thickness were evaluated using a 1–3 rating scale.

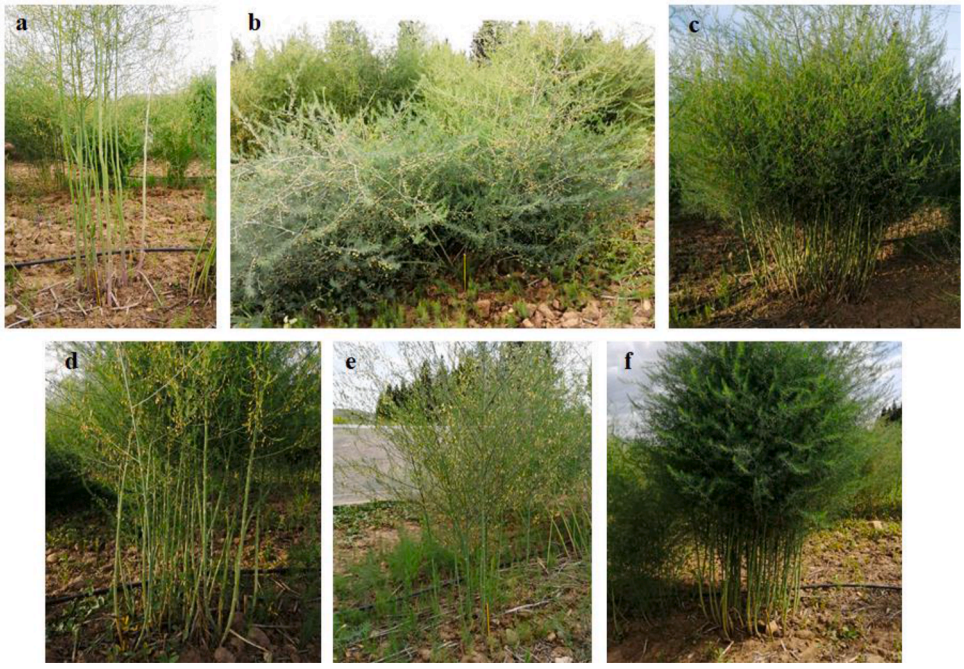

Fig. 3. Pictures of plants included in the field trial conducted to evaluate the population ($n = 1000$) obtained in this study. Differences for branching height (a, d versus b,c), stalk number (c,d,f versus a,e) and stalk thickness (a versus b,e) are displayed.

Table 5
Mean and variation coefficient (CV) of morpho-agronomic traits evaluated in an hexaploid population of cultivated asparagus with introgressions from different wild relative species (*A. pseudoscaber, A. brachyphyllus, A. macrorrhizus, A. maritimus*). Data from a set of selected plants ($n = 80$) are also presented.

Characters[a]	Population	Years	N	Mean±SE	CV (%)
Spring					
Spear number	Total	2017	943	4.8 ± 0.12	76.5
		2018	940	7.2 ± 0.22	92.6
	Selected	2017	80	7.6 ± 0.43	50.6
		2018	80	14.9 ± 0.96	57.8
Phenological state	Total	2017	943	2.4 ± 0.04	47.9
		2018	940	1.6 ± 0.03	64.2
	Selected	2017	80	3.0 ± 0.10	31.1
		2018	80	2.1 ± 0.11	48.3
Autumn					
Stalk number	Total	2016	945	1.5 ± 0.01	20.9
		2017	942	1.6 ± 0.01	24.2
	Selected	2016	80	1.7 ± 0.04	19.1
		2017	80	2.1 ± 0.05	20.6
Branching height	Total	2016	945	1.4 ± 0.01	15.8
		2017	942	1.4 ± 0.01	14.5
	Selected	2016	80	1.6 ± 0.02	11.3
		2017	80	1.5 ± 0.02	9.9
Stalk thickness	Total	2016	945	1.2 ± 0.01	18.3
		2017	942	1.2 ± 0.01	16.2
	Selected	2016	80	1.4 ± 0.02	17.2
		2017	80	1.5 ± 0.02	11.8

[a] Spear number per plant and phenological stage were evaluated following a 0–4 rating scale (0 = no spears, 1 = spears < 20 cm, 2 = spears >20 cm without primary branches, 3 = spears with primary branches and 4 = spears with secondary branches); Stalk number, branching height and stalk thickness were evaluated using a 1–3 rating scale.

The development of polyploid cultivars could contribute to broadening the diversity of the crop. There are some asparagus breeding programs that have developed polyploid cultivars already. 'Dulce Verde' (Wehner, 2002), 'Purple Passion' (Benson et al., 1996), 'Purple Pacific' (Falloon and Andersen, 1999) and 'NJ1016', among others, are tetraploid cultivars derived from the landrace 'Violetto d'Albenga'. And 'HT801' is an octoploid cultivar bred from the tetraploid landrace 'Morado de Huétor' (Moreno et al., 2012). These tetraploid landraces could be considered examples of natural introgressions, coupled with a domestication process, from hexaploid *A. maritimus* into diploid cultivated asparagus (Moreno et al., 2008; Riccardi et al., 2011). These examples reinforce the idea that crossing at different ploidy levels is possible in asparagus. In this study, it has been possible to introgress germplasm from the dodecaploid species *A. macrorrhizus* to the hexaploid population A wide range of ploidy levels (from 2*x* to 12*x*) has been found in *Asparagus* genus and most CWR species are polyploid (Bozzini, 1959; Castro et al., 2013; Valdes, 1980). Also, wild polyploid populations (4x, 8x, 10x) have been reported in *A. officinalis* (Mousavizadeh et al., 2016). Hence, more asparagus breeding efforts at different ploidy levels should be performed in this crop.

Tetraploid plants can be obtained by 6x * 2x crosses. In a previous work, some tetraploid plants were obtained making crosses between plants having that difference in ploidy levels (Moreno et al., 2010b). A collection of diploid plants with introgressions from tetraploid 'Morado de Huétor' was obtained in our breeding program (Castro et al., 2014). In addition, dihaploid plants from tetraploid male plants obtained by anther in vitro culture have been reported (Regalado et al., 2016; Riccardi et al., 2011). Therefore, our hexaploid population could be used to introgress germplasm from CWR at both tetraploid and diploid levels. Introgressive breeding followed by successive backcrossing, has been carried out to a great effect in a number of crop species such as canola (Snowdon, 2007), cultivated potato (Louwes et al., 1992) or wheat (Jones et al., 1995), among others. Therefore, the base population that, in the future, will be obtained from the selected plants could be used for developing hexaploid cultivars or for broadening the variability of tetraploid and diploid cultivars.

The wild accessions employed as donors in the hexaploid population have not been previously evaluated for biotic or abiotic stresses. Nevertheless, some of these species might be a source of new genes of interest for asparagus breeding. In this sense, resistance to both rust and virus was found in *A. maritimus* (Nothnagel et al., 2014). *Asparagus macrorrhizus* grows very close to the seaboard and this species may supposedly provide genes controlling tolerance to salinity (Regalado et al., 2017). Also, high content of saponins was found in the wild asparagus accessions that were employed as donors in the population developed in this study (Jaramillo-Carmona et al., 2017). According to the results obtained in the current study, favorable heterosis at diploid level could be explored in the near future if we use the hexaploid population to develop a diploid collection. In asparagus, heterosis were found in crosses between inbred lines and homozygous plants (Ito and Currence, 1965; Rameau, 1990). Significant advances in asparagus plant breeding were achieved by using plants derived from different genetic stocks (Benson et al., 1996; Boonen, 1988; Knaflewski, 1996). Besides a high vigor hybrid in triploid hybrids from the crosses between two different backgrounds, the tetraploid landrace 'Morado de Huetor' and current diploid cultivars, has been reported (Moreno et al., 2010c). 'Morado de Huetor' is a landrace with natural introgression from *A. maritimus* (Moreno et al., 2008)

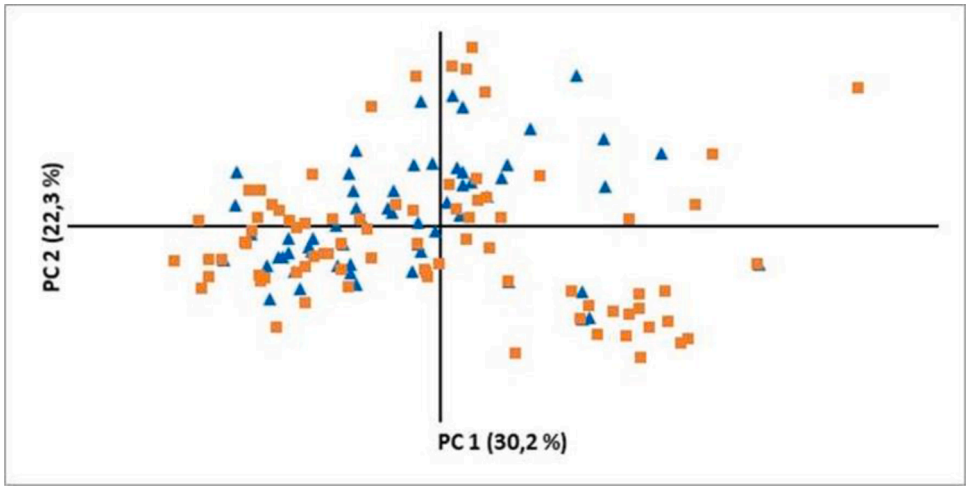

Fig. 4. PCoA analysis based on profiles from EST-SSR markers analyzed in new hexaploid population selected based on morpho-agronomic traits performance (■) and by random sampling (▲).

Breeding efforts at polyploid level remain largely unexplored in asparagus. Important asparagus morpho-agronomic traits have been described in different polyploid genetic resources. For instance, thick spears were described in the tetraploid landrace 'Violetto d'Albenga' (Branca, 1997), and high concentration in bioactive compounds (polyphenols and saponins) were reported in the landrace 'Morado de Huétor' (Fuentes-Alventosa et al., 2008; Vázquez-Castilla et al., 2013). It should be highlighted that some of the most important crops cultivated in the world are polyploids (Sattler et al., 2016). So, it seems reasonable to develop polyploid asparagus cultivars. The development of triploid hybrids between tetraploid 'Morado de Huétor' and diploid cultivars have been explored by Moreno et al., 2010c. These authors obtained heterosis in triploid hybrids even though wide variation on fertility (from high to very low) was found among tetraploid females. These results support the idea that the development of asparagus polyploid varieties could enlarge the genetic base of the asparagus cultivars.

In conclusion, the new hexaploid population we report contains greatly expanded genetic diversity and this high variation can form the basis for future progress. Our results indicate that the new hexaploid population has greater and different genetic pool than the current cultivars. Also, the method used in this work is an effective method for expanding the narrow genetic base of asparagus. On the other hand, it has been reported that asparagus plants with a higher ploidy level are more tolerant to heat than diploid plants (Chen et al., 2020). The use of plant material with a higher ploidy level together with the great genetic variability contributed by wild species introgressions should be a strategy considered by breeders for developing new asparagus varieties.

Author contributions

V.G., P.C., J.G. and R.M. conceived and designed the study; V.G., M. T.-D. and R.M. performed the field evaluation, lab analysis and analyzed the EST-SSR data; V.G., J.G. and R.M. statistical analysis; V.G., P.C., J.G. and R.M. drafted the manuscript; P.C. and J.G. revised the manuscript. All authors agree and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Amian, L., Rubio, J., Castro, P., Gil, J., Moreno, R., 2018. Introgression of wild relative *Asparagus* spp. germplasm into the Spanish landrace "Morado de Huétor.". Acta Hortic. 1223, 33–38. https://doi.org/10.17660/ActaHortic.2018.1223.5.

Benson, B.L., Mullen, R.J., Dean, B.B., 1996. Three new green asparagus cultivars; Apollo, Atlas and Grande and one purple cultivar, Purple Passion. Acta Hortic. 415, 59–66. https://doi.org/10.17660/ActaHortic.1996.415.8.

Boonen, P., 1988. Espárrago: variedades, investigación científica y producción comercial de la semilla en los Países Bajos. II Jornadas Técnicas Del Espárrago, pp. 267–275.

Bozzini, A., 1959. Revisione Cito-Sistematica del Genere *Asparagus* L. I: le specie di Asparagus della flora italiana e chiave analitica per la loro determinazione:(con Tavole XV-XVII). Caryologia 12, 199–264. https://doi.org/10.1080/00087114.1959.10797078.

Branca, F., 1997. Confronto tra genotipi di asparago in Sicilia. Inf. Agrar. 53, 43–45.

Caruso, M., Federici, C.T., Roose, M.L., 2008. EST-SSR markers for asparagus genetic diversity evaluation and cultivar identification. Mol. Breed. 21, 195–204. https://doi.org/10.1007/s11032-007-9120-z.

Castro, P., Gil, J., Cabrera, A., Moreno, R., 2013. Assessment of genetic diversity and phylogenetic relationships in *Asparagus* species related to *Asparagus officinalis*. Genet. Resour. Crop Evol. 60, 1275–1288. https://doi.org/10.1007/s10722-012-9918-3.

Castro, P., Rubio, J., Gil, J., Moreno, R., 2014. Introgression of new germplasm in current diploid cultivars of garden asparagus from a tetraploid spanish landrace "Morado de Huétor. Sci. Hortic. (Amsterdam). 168, 157–160. https://doi.org/10.1016/j.scienta.2014.01.007.

Chen, H., Lu, Z., Wang, J., Chen, T., Gao, J., Zheng, J., Zhang, S., Xi, J., Huang, X., Guo, A., Yi, K., 2020. Induction of new tetraploid genotypes and heat tolerance assessment in*Asparagus officinalis* L. Sci. Hortic. (Amsterdam). 264, 109168 https://doi.org/10.1016/j.scienta.2019.109168.

Clifford, H.T., Conran, J.G., 1987. Asparagaceae. Flora of Australia. Australian Government Publishing Service, pp. 159–164.

Dempewolf, H., Eastwood, R.J., Guarino, L., Khoury, C.K., Müller, J.V., Toll, J., 2014. Adapting agriculture to climate change: a global initiative to collect, conserve, and use crop wild relatives. Agroecol. Sustain. Food Syst. 38, 369–377. https://doi.org/10.1080/21683565.2013.870629.

Ellison, J.H., 1986. Asparagus breeding. In: Bassett, M. (Ed.), Breeding Vegetables Crops. AVI Publishing Company, Westport, pp. 521–569.

Falavigna, A., Fantino, M., 1985. Peculiar characters of the asparagus cv. "Violetto d'Albenga" grown in Italy. In: Lougheed, E., Tiessen, H. (Eds.), Proceedings Sixth International Asparagus Symposium. Guelph. University of Guelph, p. 398.

Falloon, P.G., Andersen, A.M., 1999. Breeding purple Asparagus from tetraploid "Violetto d'Albenga. Acta Hortic. 479, 109–114. https://doi.org/10.17660/ActaHortic.1999.479.13.

FAO, 2021. FAOSTAT. http://www.fao.org/statistics/en/ (accessed 3.10.21).

Fuentes-Alventosa, J.M., Jaramillo, S., Rodríguez-Gutiérrez, G., Cermeño, P., Espejo, J. A., Jiménez-Araujo, A., Guillén-Bejarano, R., Fernández-Bolaños, J., Rodríguez-Arcos, R., 2008. Flavonoid profile of green asparagus genotypes. J. Agric. Food Chem. 56, 6977–6984. https://doi.org/10.1021/jf8009766.

Geoffriau, E., Denoue, D., Rameau, C., 1992. Assessment of genetic variation among asparagus (*Asparagus officinalis* L.) populations and cultivars: agromorphological and isozymic data. Euphytica 61, 169–179.

Hajjar, R., Hodgkin, T., 2007. The use of wild relatives in crop improvement: a survey of developments over the last 20 years. Euphytica 156, 1–13. https://doi.org/10.1007/s10681-007-9363-0.

Ito, P.J., Currence, T.M., 1965. Inbreeding and heterosis in asparagus. Proceeding Am. Soc. Hortic. Sci. 86, 338–346.

Jaramillo-Carmona, S., Rodriguez-Arcos, R., Jiménez-Araujo, A., López, S., Gil, J., Moreno, R., Guillén-Bejarano, R., 2017. Saponin profile of wild *Asparagus* species. J. Food Sci. 82, 638–646. https://doi.org/10.1111/1750-3841.13628.

Jones, S.S., Murray, T.D., Allan, R.E., 1995. Use of alien genes for the development of disease resistance in wheat. Annu. Rev. Phytopathol. 33, 429–443. https://doi.org/10.1146/annurev.py.33.090195.002241.

Kanno, A., Yokoyama, J., 2011. Asparagus. In: Kole, C. (Ed.), Wild Crop Relatives: Genomic and Breeding Resources: Vegetables. Springer, Berlin, Heidelberg, pp. 23–42. https://doi.org/10.1007/978-3-642-20450-0.

Kloda, J.M., Dean, P.D.G., Maddren, C., MacDonald, D.W., Mayes, S., 2008. Using principle component analysis to compare genetic diversity across polyploidy levels within plant complexes: an example from British Restharrows (*Ononis spinosa* and *Ononis repens*). Heredity (Edinb) 100, 253–260. https://doi.org/10.1038/sj.hdy.6801044.

Knaflewski, M., 1996. Genealogy of asparagus cultivars. Acta Hortic. 415, 87–91. https://doi.org/10.17660/ActaHortic.1996.415.13.

Komarov, V., Shishkin, B., Bobrov, E., 1935. Flora of the USSR. Moscow and Leningrad.

Kubota, S., Konno, I., Kanno, A., 2012. Molecular phylogeny of the genus *Asparagus* (Asparagaceae) explains interspecific crossability between the garden asparagus (*A. officinalis*) and other *Asparagus* species. Theor. Appl. Genet. 124, 345–354. https://doi.org/10.1007/s00122-011-1709-2.

López Anido, F., Firpo, I.T., García, S.M., Cravero, V., Gatti, I., Cointry, E., 2000. Evaluación de poblaciones locales, variedades e híbridos experimentales de espárrago violeta. Hortic Argent 19, 49.

Louwes, K.M., Hoekstra, R., Mattheij, W.M., 1992. Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (St. Theor. Appl. Genet. 84, 362–370. https://doi.org/10.1007/BF00226534.

McCouch, S., Baute, G.J., Bradeen, J., Bramel, P., Bretting, P.K., Buckler, E., Burke, J.M., Charest, D., Cloutier, S., Cole, G., Dempewolf, H., Dingkuhn, M., Feuillet, C., Gepts, P., Grattapaglia, D., Guarino, L., Jackson, S., Knapp, S., Langridge, P., Lawton-Rauh, A., Lijua, Q., Lusty, C., Michael, T., Myles, S., Naito, K., Nelson, R.L., Pontarollo, R., Richards, C.M., Rieseberg, L., Ross-Ibarra, J., Rounsley, S., Hamilton, R.S., Schurr, U., Stein, N., Tomooka, N., van der Knaap, E., van Tassel, D., Toll, J., Valls, J., Varshney, R.K., Ward, J., Waugh, R., Wenzl, P., Zamir, D., 2013. Feeding the future. Nature 499, 23–24. https://doi.org/10.1038/499023a.

Mercati, F., Riccardi, P., Harkess, A., Sala, T., Abenavoli, M.R., Leebens-Mack, J., Falavigna, A., Sunseri, F., 2015. Single nucleotide polymorphism-based parentage

analysis and population structure in garden asparagus, a worldwide genetic stock classification. Mol Breed 35, 59. https://doi.org/10.1007/s11032-015-0217-5.
Moreno, R., Carmona, E., Lopez-Encina, C., Rubio, J., Gil, J., 2010a. Aplicación de marcadores microsatélites en la mejora del espárrago. Actas de Hortic 55, 221–222.
Moreno, R., Castro, P., Rubio, J., Rodríguez-Arcos, R., Gil, J., 2012. Desarrollo de una nueva variedad de espárrago octoploide "HT-801.". Actas Hortic 60, 105–108.
Moreno, R., Castro, P., Vrána, J., Kubaláková, M., Cápal, P., García, V., Gil, J., Millán, T., Doležel, J., 2018. Integration of genetic and cytogenetic maps and identification of sex hromosome in garden asparagus (*Asparagus officinalis* L.). Front. Plant Sci. 9, 1–10. https://doi.org/10.3389/fpls.2018.01068.
Moreno, R., Espejo, J.A., Cabrera, A., Gil, J., 2008. Origin of tetraploid cultivated asparagus landraces inferred from nuclear ribosomal DNA internal transcribed spacers' polymorphisms. Ann. Appl. Biol. 153, 233–241. https://doi.org/10.1111/j.1744-7348.2008.00254.x.
Moreno, R., Espejo, J.A., Cabrera, A., Millán, T., Gil, J., 2006. Ploidic and molecular analysis of "Morado de Huetor" asparagus (*Asparagus officinalis* L.) population; a Spanish tetraploid landrace. Genet. Resour. Crop Evol. 53, 729–736. https://doi.org/10.1007/s10722-004-4717-0.
Moreno, R., Espejo, J.A., Gil, J., 2010c. Development of triploid hybrids in asparagus breeding employing a tetraploid landrace. Euphytica 173, 369–375. https://doi.org/10.1007/s10681-009-0103-5.
Moreno, R., Rubio, J., Gil, J., 2010b. Transferencia de genes entre la variedad local de espárrago tetraploide Morado de Huétor y las variedades comerciales diploides. Actas de Hortic 55, 219–220.
Mousavizadeh, S.J., Hassandokht, M.R., Gil, J., Millan, T., Moreno, R., 2018. Assessment of genetic diversity in Iranian *Asparagus* spp. related to garden asparagus. Acta Hortic. 1223, 39–44.
Mousavizadeh, S.J., Hassandokht, M.R., Kashi, A., 2015. Multivariate analysis of edible *Asparagus* species in Iran by morphological characters. Euphytica 206, 445–457. https://doi.org/10.1007/s10681-015-1508-y.
Mousavizadeh, S.J., Hassandokht, M.R., Kashi, A., Gil, J., Cabrera, A., Moreno, R., 2016. Physical mapping of 5S and 45S rDNA genes and ploidy levels of Iranian *Asparagus* species. Sci. Hortic. (Amsterdam). 211, 269–276. https://doi.org/10.1016/j.scienta.2016.09.011.
Nothnagel, T., Budahn, H., Krämer, I., Schliephake, E., Schreyer, L., Krämer, R., 2014. Resistance to Asparagus virus 1 in the wild relative *Asparagus amarus*. J. Phytopathol. 162, 180–189. https://doi.org/10.1111/jph.12169.
Peakall, R., Smouse, P.E., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research an update. Bioinformatics 28, 2537–2539. https://doi.org/10.1093/bioinformatics/bts460.
Prohens, J., Gramazio, P., Plazas, M., Dempewolf, H., Kilian, B., Díez, M.J., Fita, A., Herraiz, F.J., Rodríguez-Burruezo, A., Soler, S., Knapp, S., Vilanova, S., 2017. Introgressiomics: a new approach for using crop wild relatives in breeding for adaptation to climate change. Euphytica 213. https://doi.org/10.1007/s10681-017-1938-9.
Rameau, C., 1990. Fifteen years of experiments on asparagus F1 hybrids: synthesis, evaluation of homozygous parents and application to the French breeding programme. Acta Horticulturae. International Society for Horticultural Science (ISHS), Leuven, Belgium, pp. 47–54. https://doi.org/10.17660/ActaHortic.1990.271.4.
Regalado, J.J., Martín, E.C., Madrid, E., Moreno, R., Gil, J., Encina, C.L., 2016. Production of "super-males" of asparagus by anther culture and its detection with SSR-ESTs. Plant Cell. Tissue Organ Cult. 124, 119–135. https://doi.org/10.1007/s11240-015-0880-6.
Regalado, J.J., Moreno, R., Castro, P., Carmona-Martin, E., Rodríguez, R., Pedrol, J., Larrañaga, N., Guillén, R., Gil, J., Encina, C.L., 2017. *Asparagus macrorrhizus* Pedrol, Regalado et López-Encina, an endemic species from Spain in extreme extinction risk, is a valuable genetic resource for asparagus breeding. Genet. Resour. Crop Evol. 64, 1581–1594. https://doi.org/10.1007/s10722-016-0456-2.
Riccardi, P., Casali, P.E., Mercati, F., Falavigna, A., Sunseri, F., 2011. Genetic characterization of asparagus doubled haploids collection and wild relatives. Sci. Hortic. (Amsterdam). 130, 691–700. https://doi.org/10.1016/j.scienta.2011.08.028.
Sattler, M.C., Carvalho, C.R., Clarindo, W.R., 2016. The polyploidy and its key role in plant breeding. Planta 243, 281–296. https://doi.org/10.1007/s00425-015-2450-x.
Snowdon, R.J., 2007. Cytogenetics and genome analysis in brassica crops. Chromosom. Res. 15, 85–95. https://doi.org/10.1007/s10577-006-1105-y.
The Plant List, 2013. http://www.theplantlist.org/ (accessed 9.1.21).
Torres, A.M., Weeden, N.F., Martín, A., 1993. Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. Theor. Appl. Genet. 85, 937–945. https://doi.org/10.1007/BF00215032.
Valdes, B., 1980. Asparagus. In: Tutin, T., Heywood, V., Burges, N., Valentine, D. (Eds.), Flora Europaea. Cambridge, pp. 71–73.
Vázquez-Castilla, S., Jaramillo-Carmona, S., Fuentes-Alventosa, J.M., Jiménez-Araujo, A., Rodríguez-Arcos, R., Cermeño-Sacristán, P., Espejo-Calvo, J.A., Guillén-Bejarano, R., 2013. Saponin profile of green asparagus genotypes. J. Agric. Food Chem. 61, 11098–11108. https://doi.org/10.1021/jf403343a.
Venezia, A., Soressi, G., Falavigna, A., 1993. Aspects related to utilization of wild *Asparagus* species in Italy. Agric Ric 141, 41–48.
Wehner, T.C., 2002. Vegetable Cultivar Descriptions for North America List 26 2002. HortScience 37, 15–78. https://doi.org/10.21273/hortsci.48.2.245.
Xinqi, C., Tamanian, K., 2000. Asparagus. In: Zheng-yi, W., Raven, P.H. (Eds.), Flora of China: Flagellariaceae Through Marantaceae. Science and MO Botanical Garden, St. Louis, MO, USA, pp. 209–216.