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Original article

New hybrids between cultivated and wild species of asparagus (*Asparagus* spp.) and their validation by SSR markers

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Summary

Garden asparagus (*Asparagus officinalis* L., $2n = 2x = 20$) is an important vegetable crop cultivated worldwide. A narrow genetic base has been pointed out in the current cultivars. Wild related asparagus species could be a valuable resource to extend genetic variation of this crop, but their use have been scarce so far. In this study, two new interspecific hybrid plants between cultivated and wild asparagus species, *A. persicus* (2*x*) and *A. breslerianus* (8*x*), were obtained by hand-pollination. The genetic origin of hybrid plants was verified by SSR molecular markers. Besides, diploid hybrids between cultivated asparagus and both *A. persicus* and a wild accession of *A. officinalis* were detected by SSR markers in progenies obtained from open pollination. In this case, the genetic polymorphism among 21 asparagus accessions belong to 10 dioecious species (*A. officinalis* (2*x*, 4*x*, 8*x*, 10*x*), *A. verticillatus* (2*x*), *A. breslerianus* (8*x*), *A. brachyphyllus* (6*x*), *A. pseudoscaber* (6*x*), *A. acutifolius* (4*x*), *A. persicus* (2*x*), *A. macrorrhizus* (12*x*), *A. maritimus* (6*x*) and *A. prostratus* (4*x*)) grown in a field collection under open pollination condition were analyzed by using 26 SSR markers. Specific bands and specific absences of amplicon were found among the accessions. The utility rate of this information for detecting of hybrid plants was tested by employing progenies from the diploid species included *A. persicus*, wild accession of *A. officinalis* and *A. verticillatus*. Fifteen and nine hybrid plants were detected in progenies from *A. persicus* and wild accession of *A. officinalis* respectively, whereas no interspecific plants were found in the progeny from *A. verticillatus*. The flow cytometry analysis revealed a diploid level for all these 24 hybrid plants. The male parents of these hybrids could be detected by employing two polymorphic markers (AG11 and asp_c14231) among diploid accessions which applied in this study. The 15 hybrid plants detected in the progeny from *A. persicus* were identified as interspecific hybrids with *A. officinalis*, of which 10 of them were derived from *A. persicus* × *A. officinalis* (naturalized) cross and 5 hybrid plants were related to *A. persicus* × *A. officinalis* (wild) cross. The 9 detected hybrids in the progeny from wild accession of *A. officinalis* were intraspecific with cultivated *A. officinalis.* With the aim to develop new asparagus germplasm, a backcrossing program toward cultivated asparagus has been recently initiated in our plant breeding project by employing the hybrid plants obtained in this study.

Significance of this study

What is already known on this subject?

- The genetic base of garden asparagus is narrow. Thus, the development of new germplasm is a major goal in asparagus breeding. With this aim, crop wild relatives could be useful, but these genetic resources have largely remained unexploited in this crop.

What are the new findings?

- New hybrids between cultivated and wild plants belonging to both wild population of the cultivated species (*A. officinalis*) and two wild species (*A. persicus*, *A. breslerianus*) have been obtained. Effective molecular tools to search for hybrid plants in progenies of females growing in an experimental field containing genetic resources under open pollination have also been developed.

What is the expected impact on horticulture?

- The plant material obtained in this study may be useful in the development of new asparagus germplasm. Other different interspecific hybrids obtained in the present study could be detected in the future in the progenies of females growing in an experimental trial under open pollination established with genetic resources of this crop.

Keywords
interspecific hybrids, wild asparagus, SSR markers, genetic resources

Introduction

Asparagus is a genus member of the Liliaceae family that comprises three subgenera including dioecious (*Asparagus* subgenus) and hermaphroditic species (*Protasparagus* and *Myrsiphyllum* subgenus) (Clifford and Conran, 1987). This genus includes over 200 species distributed in the arid and sub-arid regions of Europe, Asia, Africa, and Australia (Kubituki and Rudall, 1998). A basic chromosome number of x=10 is proposed across *Asparagus* spp. and polyploidization phenomenon may have played an important role in the evolution of this genus because different ploidy levels have been reported at both specific and intraspecific taxa (Moreno et al., 2006; Kanno and Yokoyama, 2011; Mousavizadeh et al., 2016).

Asparagus officinalis L. ($2n = 2x = 20$) is a dioecious species widely cultivated as a vegetable crop and the most econom-

ically important asparagus species worldwide. Most current cultivars have a common genetic origin, a Dutch population (S.XVIII) known as 'Violet Dutch' (Geoffriau et al., 1992; Knaflewski, 1996). This fact may explain the narrow genetic base of this crop as it has been revealed in different studies employing both isozyme (Brettin and Sink, 1992; Geoffriau et al., 1992) and DNA molecular markers (Khandka et al., 1996; Mercati et al., 2015; Moreno et al., 2006). To enlarge the genetic base of the asparagus cultivars, wild *Asparagus* spp. can be employed. Different agronomic traits of interest have been described in wild *Asparagus* spp. such as resistance to important biotic or abiotic stresses (Kanno and Yokoyama, 2011; Nothnagel et al., 2012) or higher profiles in bioactive compounds (Ferrara et al., 2011; Vázquez-Castilla et al., 2013; Jaramillo-Carmona et al., 2017).

In the current scenario of climate change and in order to develop new cultivars adapted to new environmental conditions an increase of genetic diversity must be considered in breeding programs. Recently a new approach, called 'introgressiomics', has been proposed (Prohens et al., 2017). It consists of mass scale development of plant materials and populations with introgressions from crop wild relatives into the genetic background of crops. Recent successful examples are found for drought tolerance in safflower (Espanani et al., 2019) or yield in soya (Hegstad et al., 2019).

In asparagus, the first interspecific hybridization was reported between the cultivated asparagus and a wild species (*A. davuricus* Fish. ex Link) which was carried out by Norton (1913). Since then, interspecific hybridizations between *A. officinalis* and wild *Asparagus* spp. belonging to three subgenera have been carried out, but only successful crosses were obtained with some species included in the *Asparagus* subgenus so far (Kanno and Yokoyama, 2011). An asparagus tetraploid landrace 'Morado de Huetor' (4*x*) was reported as natural interspecific hybrid between cultivated asparagus and the wild relative species *A. maritimus* (L.) Mill. (Moreno et al., 2008). This landrace was used to introgress its germplasm in current diploid (2*x*) cultivars (Castro et al., 2014) and has been used as a genetic bridge to transfer germplasm from different European wild species to the crop (Amian et al., 2018; Garcia et al., 2021).

The species of *Asparagus* subgenus are mostly distributed in the temperate climate regions of Europe and Asia, and the proposed centre of origin of this crop comprises Eastern Europe, Caucasus, and Siberia (Sturtevant, 1890). To date, interspecific hybrids have been obtained with wild related species that have an European distribution (*A. maritimus, A. pseudoscaber* Grec., *A. tenuifolius* Lam., *A. prostratus* Dum., *A. brachyphyllus* Turcz., *A. acutifolius* L., *A. macrorrhizus* Pedrol, Regalado et López-Encina) (Castro et al., 2013) and with others whose geographical distribution comprises regions of the far East (*A. davuricus*, *A. oligoclonos* Maxim., *A. schoberoides* Kunth, *A. kiusianus* Makino) (Kanno and Yokoyama, 2011; Ito et al., 2011). In this region other *Asparagus* subgenus species such as *A. persicus* Baker, *A. breslerianus* Schult. & Schult. f., or *A. verticillatus* L. can also be found. However, except *A. verticillatus*, no attempts to cross these species with the cultivated one have been carried out so far. In the case of *A. verticillatus* the crosses were unsuccessful to obtain interspecific hybrids (Ito et al., 2008).

Different factors such as post-zygotic barriers or large differences in the ploidy level of the parents employed in the crosses can make hybridization impossible. In this sense, sometimes the employment of a genetic bridge might be helpful to overcome these cross barriers. So that Falavigna et al. (2008) obtained one hybrid plant from the cross of a tetraploid plant, derived from *A. officinalis* × *A. maritimus*, with *A. acutifolius* (4*x*). As it has been mentioned above the tetraploid landrace 'Morado de Huetor' was also used as bridge to develop new hexaploid (6*x*) germplasm with introgression of wild relative 6*x* and 12*x* species (Amian et al., 2018; Garcia et al., 2021).

Morphological characters are not always suitable to validate the hybrid nature of plants obtained from crosses carried out in the plant breeding programs. Nowadays, molecular markers are commonly used to assess the hybrid nature of cross derived plants (Pereira et al., 2016; Caballo et al., 2018). In asparagus, Valente et al. (2009) reported the use of RAPD markers in the assessment of genomic introgression of *A. acutifolius* and *A. maritimus* into *A. officinalis*. Similarly, Castro et al. (2014) assessed the presence of germplasm of 'Morado de Huétor' into diploid *A. officinalis* by the employment of microsatellite markers (SSRs). Microsatellite markers or Simple sequence repeats (SSRs) are low-cost, reproducible between labs, usually easily detectable, and generate a high level of polymorphism between tested genotypes. In asparagus, these markers have been employed in several studies including the assessment of the genetic diversity of *A. officinalis* cultivars and different genetic resources of this crop (Caruso et al., 2008; Castro et al., 2013; Li et al., 2016; Garcia et al., 2021; Mousavizadeh et al., 2021), detecting of somaclonal variation (Regalado et al., 2015) and genetic mapping (Takeuchi et al., 2012; Mercati et al., 2013; Moreno et al., 2018).

In this study, interspecific crosses were done by employing wild asparagus species (*A. persicus* (2*x*), *A. breslerianus* (8*x*), *A. verticillatus* (2*x*)) and both wild and cultivated *A. officinalis* as a first step of introgression of new germplasm into cultivated ones. Moreover, the efficiency of SSR markers to verify the hybrid origin of plants obtained from both controlled hand-pollination and open-pollination was also studied.

Materials and methods

Production of interspecific hybrid plants between wild asparagus species (*A. persicus* and *A. breslerianus*) and cultivated plants. Validation of hybrid origin by EST-SSR markers

Two diploid female plants (DeP_1 and AOD_1) of *A. officinalis* derived respectively from the cultivar 'De Paoli' and an Iranian naturalized population from Karaj (Iran) were hand pollinated with a male plant (ApeM_1) of *A. persicus* (2*x*). Besides, a female octoploid plant (HT167) detected in the tetraploid landrace 'Morado de Huétor' (Moreno et al., 2006) was employed in a cross carried out with a male plant (ABY_1) of *A. breslerianus* (8*x*). To prevent pollinations from unknown pollen sources, the female parents were previously bagged.

Two EST-SSR markers (AG7 and AG8) developed in garden asparagus by Caruso et al. (2008) were employed to verify the interspecific origin of the plants that may be derived from these crosses. Total genomic DNA was isolated from 1 g of tips of young spears following a modified CTAB extraction protocol (Doyle and Doyle, 1990). The quantity and quality of the isolated DNA was evaluated by spectrophotometric absorbance at 260 nm (NanoDrop ND-1000; Thermo Scientific, Waltham). Forward primers were synthesized with fluorescent dyes (6-FAM or Hex). The PCR amplifications were performed in a 10-µL reaction mixture containing 20 ng of

bulked genomic DNA, 5X polymerase buffer, 2 mM $MgCl_2$, 0.8 mM of dNTP, 0.25 µM of forward primer (HEX or 6-FAM), 0.25 µM of reverse primer, and 0.05 U of Taq polymerase (Promega). Amplification cycles were performed in a Perkin-Elmer Cetus DNA Thermal Cycler (9600) programmed as follows: (A) 1 min denaturation at 94°C, (B) 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerisation at 72°C for 1.5 min; (C) followed by a 10-min final extension at 72°C.

The PCR products were separated using an automated capillary sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems/Hitachi, Madrid, Spain) in the Unit of Genomics of the Central Research Support Service at the University of Córdoba. The size of the amplified bands was calculated based on an internal DNA standard (400HD-ROX) with GeneScan software (v. 3.x) and the results were interpreted using the Genotyper program (v. 3.7), all from Applied Biosystems.

EST-SSR markers to search for interspecific hybrids in progenies from accessions belong to an *Asparagus* ssp. collection growing in a plot under open-pollination conditions

The first step was to evaluate the genetic polymorphism of an *Asparagus* ssp. collection by SSR markers. Twenty-one accessions belong to the collection of genetic resources of the asparagus breeding program developed in the University of Cordoba (Spain) were used (Table 1). These accessions belong to 10 species of *Asparagus* subgenus including *A. officinalis* (wild and naturalized populations) and different wild species mainly distributed through Europe (*A. macrorrhizus*, *A. maritimus*, *A. pseudoscaber*, *A. prostratus*, *A. acutifolius*) and Asia (*A. verticillatus*, *A. brachyphyllus*, *A. persicus*, *A. breslerianus*) (Table 1). Besides in this study a tetraploid landrace ('Morado de Huétor') was included for which an interspecific origin (*A. officinalis* × *A. maritimus*) is proposed (Moreno et al., 2008). The accession AOW was received from Yorshkar-Ola (Russia) as a wild *A. officinalis* population, however, it resembles more like a cultivated asparagus and probably it was a naturalized population. The plot containing all these genetic resources was established in spring 2015. Twenty plants per accession were used in two neighbor rows 1.5 m apart and 0.5 m between plants within row. The distance between neighbor rows was 2 m (Figure 1).

To search for specific alleles in this collection a set of 26 EST-SSR markers was employed to assess the genetic polymorphism among accessions. These markers were developed in garden asparagus by Caruso et al. (2008) (AGA1, AG5, AG7, AG8, AG10, AG11, AG12, TC2, TC4, TC6) and Mercati et al. (2013) (asp_c10809, asp_c12534, asp_c13301, asp_c1401, asp_c14231, asp_c17381, asp_c17476, asp_c22357, asp_c2370, asp_c4789, asp_c6290, asp_c6470, asp_c7389, asp_c753, asp_c8280, asp_c923). Around ten plants were randomly chosen within each accession. This population size ($n = 10$) is enough to detect most frequent alleles ($p > 0.25$) present in each accession with a probability of 94% (Hanson, 1959). The DNA isolation of these 10 plants was carried out as two bulks containing each one equal amounts (0.2 g) of young tissue from five different plants. DNA was isolated following a modified CTAB extraction protocol (Doyle and Doyle, 1990). DNA bulks were used as DNA templates in the PCR reactions. Both PCR mix reaction and amplification conditions were similar to those which were detailed previously in this study. In this case, forward primers were not synthetized with fluorescent dyes because polymorphisms between accessions were visualized in agarose gels. For that, gels were composed of a mixture of 1% D-1 low EEO (electro-endosmosis) agarose and 1% LM-Sieve agarose (Pronadisa,

Table 1. Characteristics of *Asparagus* spp. accessions analyzed by SSR markers in this study.

Accession codes	Origin	No. of plants analyzed	Ploidy level[a]	Type	Species
AOM	Mahmood abad (Iran)	10	4*x*	Wild	*A. officinalis* L.
AOBA	Balade (Iran)	10	8*x*	Wild	*A. officinalis* L.
AOG	Gazanak (Iran)	10	8*x*	Wild	*A. officinalis* L.
AOT	Taleghan (Iran)	10	4*x*	Wild	*A. officinalis* L.
AOBI	Bayanloo (Iran)	2	10*x*	Wild	*A. officinalis* L.
AOK	Kerman (Iran)	10	2*x*	Wild	*A. officinalis* L.
AOW	Yorshkar-Ola (Russia)	10	2*x*	Naturalized	*A. officinalis* L.
AOD	Karaj (Iran)	10	2*x*	Naturalized	*A. officinalis* L.
AOU	Utrera (Spain)	10	2*x*	Naturalized	*A. officinalis* L.
HT	Granada (Spain)	10	4*x*	Landrace	*A. officinalis* × *A. maritimus*
AVSH	Chalaki (Iran)	10	2*x*	Wild	*A. verticillatus* L.
AVSP	Shal (Iran)	10	2*x*	Wild	*A. verticillatus* L.
AVC	Klarood (Iran)	4	2*x*	Wild	*A. verticillatus* L.
APeM	Manjil (Iran)	10	2*x*	Wild	*A. persicus* Baker
ABY	Yazdoo (Iran)	10	8*x*	Wild	*A. breslerianus* Schult. & Schult.f.
APB	Bares (Spain)	10	4*x*	Wild	*A. prostratus* Dumort
AMa	Albania	10	6*x*	Wild	*A. maritimus* (L.) Mill.
AMC	Cartagena (Spain)	10	12*x*	Wild	*A. macrorrhizus* Pedrol & al.
APSC	Brno (Czech Rep.)	10	6*x*	Wild	*A. pseudoscaber* Grec.
ABra	Yorshkar-Ola (Russia)	10	6*x*	Wild	*A. brachyphyllus* Turz.
AA	Córdoba (Spain)	10	4*x*	Wild	*A. acutifolius* L.

[a] Data of ploidy levels were obtained from Castro et al. (2013) and Mousavizadeh et al. (2016).

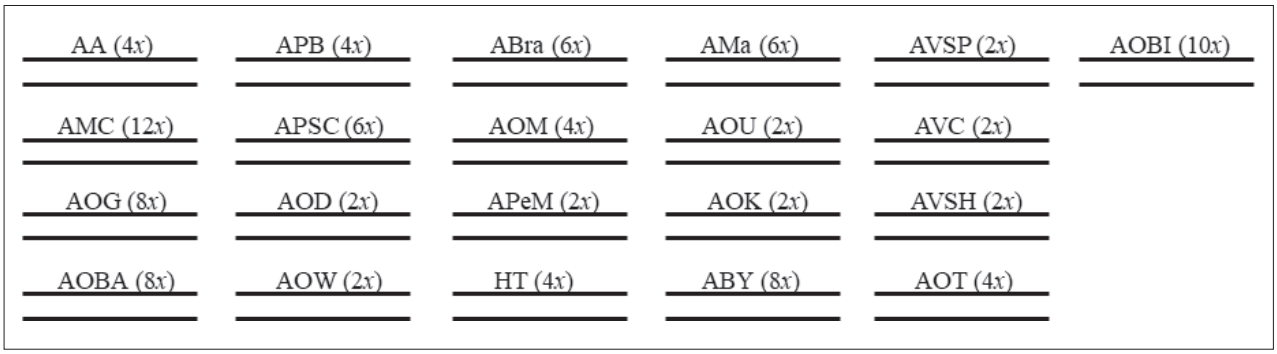

Figure 1. Field distribution of the asparagus accessions employed in this study. Each accession is planted in two neighbor rows of 5 m length. Between brackets is the ploidy level. AOBI, AOM, AOK, AOBA and AOT are wild *A. officinalis* accessions; AOU, AOD and AOW are naturalized *A. officinalis* accessions; AVSP, AVC and AVSH are wild *A. verticillatus* accessions; AA, APB, ABra, AMa, AMC, APSC, APeM and ABY are wild *A. acutifolius, A. prostratus, A. brachyphyllus*, *A. maritimus*, *A. macrorrhizus*, *A. pseudoscaber*, *A. persicus* and *A. breslerianus* accessions, respectively; HT is a tetraploid landrace of cultivated asparagus.

Madrid, Spain) in 1_TBE (trisborate EDTA) buffer. In those cases that the polymorphism was presence/absence of PCR product, a marker asp_c6470 was used as positive control to discard the presence of false negatives in the amplification.

Polymorphic EST-SSR markers were used to detect interspecific hybrids in progenies from diploid females of *A. persicus* (APeM accession), *A. verticillatus* (AVSH and AVSP accessions) and a wild *A. officinalis* (AOK accession). These accessions were growing in the field experimental plot under open pollination and the progenies were genotyped to elucidate between two alternatives of genetic origin: a) cross between plants of the same accession, or b) cross with male parents belong to different accessions or species.

In order to assess the ploidy levels of these progenies to detect hybrids from crosses with polyploid accessions the relative DNA contents were measured by using flow cytometry (PloidyAnalyser PA-I; Partec GmbH, Münster, Germany). For analysis, 0.5 cm^2 of young leaves or tips of young spears was chopped with a razor blade for 30–60 s to release nuclei in a Petri dish containing 0.4 mL of nuclei isolation buffer (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, extraction buffer). The homogenate was filtered through a 50 µm nylon mesh (Partec 50 µm CellTrics disposable filter), and subsequently nuclei were stained with fluorescent dye (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer, about 1.6 mL). The samples were then analyzed after 30 s of incubation. Spear of a diploid plant of the current diploid asparagus cultivar 'Grande' was used as reference.

Results

Thirteen flowers of two diploid *A. officinalis* female parents (AOD_1 and DeP_1) and 37 of the octoploid one (HT167) were hand-pollinated with pollen from *A. persicus* (2*x*) and *A. breslerianus* (8*x*) accessions, respectively. As results, one fruit from each cross was obtained. Each of the two fruits harvested from diploid *A. officinalis* parents produced one seed, whereas 3 seeds were separated from the fruit harvested from octoploid HT167 × *A. breslerianus* (ABY_1) cross. These 5 seeds were sown in pots of 7 × 8 × 13 cm in a greenhouse under controlled temperature (max. 27°C, min. 15°C). Only a putative interspecific plant was obtained from both AOD_1 × *A. persicus* (APeM_1) and HT167 × ABY_1 crosses whereas no plant was obtained from DeP_1 × APeM_1 cross. The hybrid origin of the two obtained plants was verified by EST-SSR markers. Parental specific alleles were detected in both plants confirming their interspecific origin (Figure 2).

The possibility to attain interspecific hybrids in a collection of *Asparagus* ssp. growing in a field plot under open pollination was explored by using progenies from diploid accessions present in this collection. For this purpose, the genetic polymorphism among the different asparagus accessions was assessed by employing SSR markers and using agarose gels. As a result, a single band per accession was obtained in most markers (21) employed in this study whereas two bands (alleles) for 5 markers were observed in some accessions (Table 2). From the total of markers, 18 were polymorphic and 13 of them showed absences of amplicon in some accessions (Table 2). Within diploid accessions, two specific bands were found in *A. persicus* for TC6 and AGA1 markers and one band in a wild accession (AOK) of *A. officinalis* for asp_c14231 (Figure 3). As it has been mentioned above no amplification was obtained for some markers in some accessions and therefore polymorphism for presence/absence of amplicon was considered. Seven specific absences were observed, one in *A. persicus* for asp_c2370, three in the three accessions of *A. verticillatus* for asp_c12877, asp_c923 and AG12, and one in the wild *A. officinalis* (AOK) for AG7 (Table 2). The specific allelic bands found could be helpful to detect hybrids derived from crosses where these accessions could be involved. In addition, the specific absences of amplicon obtained could be also useful to detect the possible hybrid origin of some plants. In this case, the presence of amplicons in some progenies will denote an interaccession hybrid nature.

In this study four different progenies derived from three diploid species: one from *A. officinalis* (AOK), two from *A. verticillatus* (AVSP and AVSH), and one from *A. persicus* (APeM), were used to detect hybrid plants. All progenies were diploids which indicated their male parent should be a diploid plant either from the same accession or from the other diploid accessions growing in the experimental plot. Three markers included AG7, asp_c12877 and TC6 were chosen to genotype the plants of the progenies. Wild *A. officinalis* (AOK) and *A. verticillatus* showed a specific absence of band for AG7 and asp_c12877 respectively, while *A. persicus* showed a specific band for TC6 (Table 2). As results, 15 of 44 (34.1%) and 9 of 310 (2.9%) genotyped progenies related to *A. persicus* and *A. officinalis* (AOK) showed a hybrid origin, respectively (Figure 4). Conversely, none of the 171 progenies derived from *A. verticillatus* showed a hybrid origin.

In order to elucidate among the male parents of the hy-

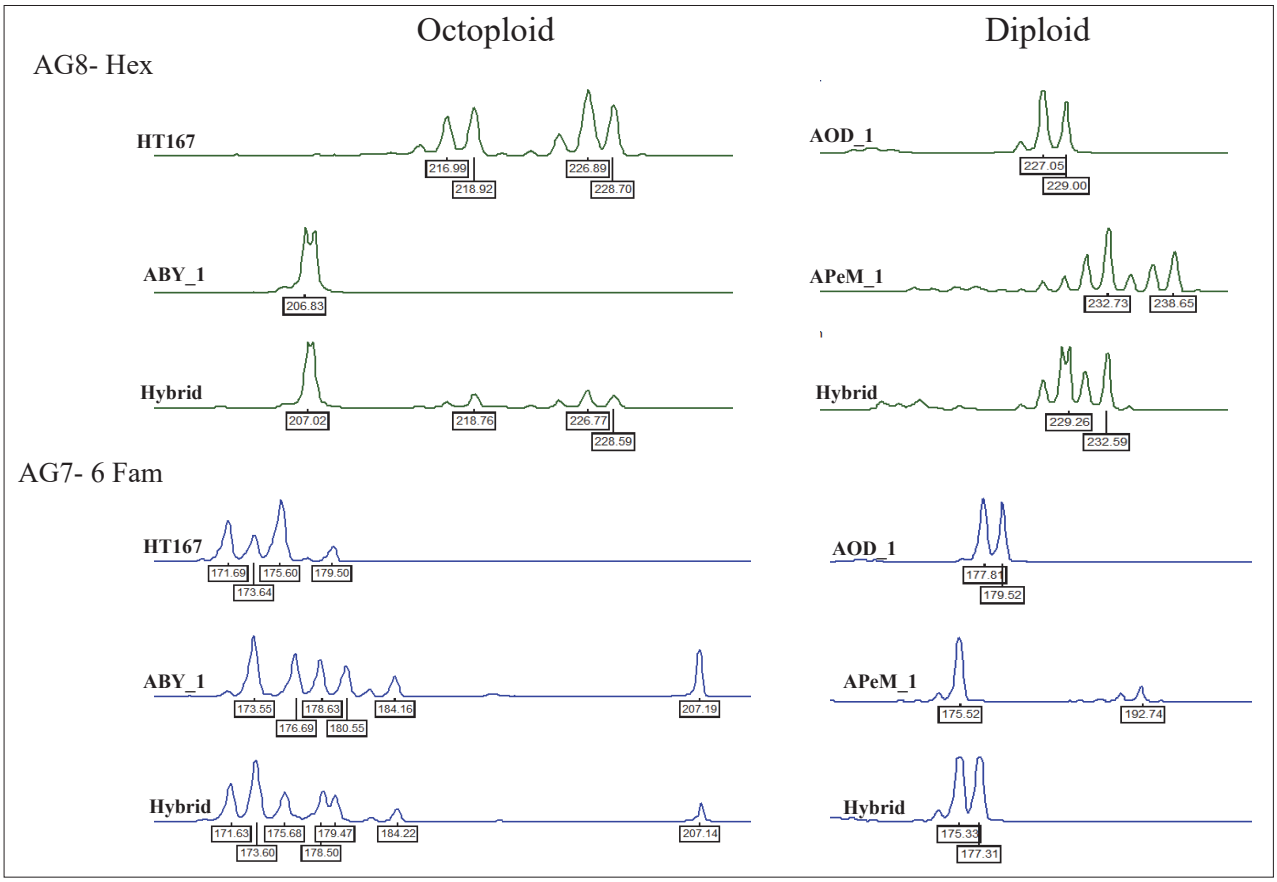

Figure 2. Genetic characterization of interspecific hybrids obtained from the crosses between octoploid plants of 'Morado de Huétor' (HT167) (female) and *A. breslerianus* (ABY_1) (male) and diploid ones of *A. officinalis* (AOD_1) (female) and *A. persicus* (APeM_1) (male) by SSR markers (AG8-Hex and AG7-6 FAM).

brids detected in the progeny, different specific bands obtained in this study could be also useful (Table 2). Hence, from the total of 15 hybrid plants detected in the progenies of *A. persicus*, five of them showed a specific band for AGA1 (605 pb) present in *A. officinalis* and *A. verticillatus* accessions and for AG11 an absent band like in the wild *A. officinalis* (AOK) and the *A. verticillatus* (AVSH) accessions (Figure 5, lane 10). Therefore, the male of these hybrids could be AOK or AVSH. However, the presence of a specific band (140 pb) for asp_c14231 from AOK in these hybrids confirms that the male parent is the wild *A. officinalis* accession. The 10 remaining hybrids showed a band for AG11 present in the three naturalized diploid *A. officinalis* accessions (AOU, AOW, AOD), which indicated each of them could be the male parent (Figure 5, lane 11). Thus, according to our results, all these 10 plants are interspecific hybrids between *A. persicus* and *A. officinalis*. About the 9 hybrid plants detected in the progenies of the wild *A. officinalis* (AOK), all of them showed the specific band of the diploid accessions of *A. officinalis* AOU, AOW and AOD for AG11 and were heterozygous for asp_c14231 (Figure 5, lane 12). Non-specific band from *A. verticillatus* was present in these progenies. This result suggests an intraspecific origin of these hybrids.

Discussion

Garden asparagus has a narrow genetic base due to the common origin proposed for the current diploid cultivars. Therefore, one of the main objectives in asparagus breeding is the development of new germplasm. According to different studies which both wild and cultivated species were conducted in them, wild asparagus populations could be a valuable genetic resource because of their different and higher genetic variability compared to asparagus commercial cultivars (Moreno et al., 2008; Castro et al., 2013; Nothnagel et al., 2017). In addition, wild asparagus species could be a source of genes or alleles which can control traits of interest or nutraceutical compounds that are in a smaller amount or absent in the cultivated species (Ferrara et al., 2011; Kanno and Yokoyama, 2011; Nothnagel et al., 2012; Vázquez-Castilla et al., 2013; Jaramillo-Carmona et al., 2017).

Despite the important role that the wild related asparagus species could have in the genetic improvement of this crop, the breeding efforts in the development of new asparagus germplasm employing this genetic resource have been scarce so far (Garcia et al., 2021). In this study two interspecific hybrids between cultivated and wild asparagus plants belonging to *A. persicus* and *A. breslerianus* species have been obtained for the first time by handmade crosses. The accession of *A. breslerianus* included in this study was collected from a region characterized by a hot and dry climate with a high salinity soil (Mousavizadeh et al., 2015). Thus, the interspecific hybrid plant obtained with *A. breslerianus* could be a source of new alleles or genes potentially useful in asparagus breeding to overcome abiotic stresses affecting the crop.

Molecular markers are a valuable tool to validate the hybrid origin as it has been shown in this work and they have also proved to be an effective tool to search for hybrid plants in progenies of females growing in an experimental field under open pollination. In our study SSR markers were used because they are low cost, reproducible between labs and they show a good transferability between *Asparagus* species (Castro et al., 2013; Nothnagel et al., 2017; Mousavizadeh et

Table 2. Polymorphism among accessions of *Asparagus* spp. for absence of amplicon (-) and/or allelic size (pb) using SSR markers.

Accession code[a]	SSR markers																	
	asp_c10809	asp_c12877	asp_c14231	asp_c22357	asp_c2370	asp_c2736	asp_c4789	asp_c7389	asp_c8280	asp_c923	AGA1	AG5	AG7	AG10	AG11	AG12	TC2	TC6
AVSP (2*x*)	178	-	155	160	180	150	-	-	182	-	605	1050	290	280	1700	-	-	1100
AVC (2*x*)	178	-	-	160	180	150	165	-	182	-	605	1050	290	280	1650	-	-	1100
AVSH (2*x*)	178	-	155	160	180	150	-	162	182	-	605	1050	290	280	-	-	-	1100
AOU (2*x*)	178	215	170	160	180	150	165	162	182	125	605	1050	290	280	1800	228	1500	1100
AOD (2*x*)	178	215	170	160	180	150	165	162	182	125	605	1050	290	280	1800	228	1500	1100
AOW (2*x*)	178	215	170	160	180	150	165	162	182	125	605	1050	290	280	1800	228	1500	1100
AOK (2*x*)	178	215	140	160	180	150	-	162	182	125	605	1050	-	280	-	228	-	1100
APeM (2*x*)	178	215	155	160	-	150	165	162	182	125	560	1050	290	280	-	228	1500	1180
APB (4*x*)	178	215	170	160	180	150	-	162	182	125	560/605	1050	290	280	1700	228	1500	1100/1180
HT (4*x*)	178	215	155	160	180	150	165	162	182	125	605	1050	290	280	1700/1800	228	1500	1100
AA (4*x*)	255	215	-	120	180	-	165	-	-	-	605	860/1050	290	-	1700/1800	-	-	1100
AOM (4*x*)	178	215	155	160	180	150	165	162	182	125	560/605	1050	290	280	1700	228	1500	1100
AOT (4*x*)	178	215	155	160	180	150	165	162	182	125	560/605	1050	290	280	-	228	1500	1100
ABra (6*x*)	178	215	170	160	180	150	165	162	182	125	605	1050	290	280	1700/1800	228	1500	1100
AMa (6*x*)	178	215	170	160	180	150	165	162	182	125	560/605	1050	290	280	1700/1800	228	1500	1100
APSC (6*x*)	178	215	170	160	180	150	165	162	182	125	605	1050	290	280	1700/1800	228	1500	1100
AOBA (8*x*)	178	215	155	160	180	150	165	162	182	125	560/605	1050	290	280	1800	228	1500	1100
AOG (8*x*)	178	215	155	160	180	150	165	162	182	125	605	1050	290	280	1700/1800	228	1500	1100
ABY (8*x*)	178	215	155	160	180	150	165	162	182	125	605	1050	290	280	1800	228	-	1100
AOBI (10*x*)	178	215	155	160	180	150	165	162	182	125	560/605	1050	290	280	1700/1800	228	1500	1100
AMC (12*x*)	178	215	155/170	160	180	150	165	162	182	125	605	1050	290	280	1700/1800	228	1500	1100

[a] AV_ are *A. verticillatus*; AO_ are *A. officinalis*; APeM_ is *A. persicus*; APB is *A. prostratus*; HT is landrace 'Morado de Huetor'; AA is *A. acutifolius*; ABra is *A. brachyphyllus*; AMa is *A. maritimus*; APSC is *A. pseudoscaber;* ABY is *A. breslerianus*; and AMC is *A. macrorrhizus.* In brackets are ploidy levels.

al., 2021). Today the next-generation sequencing facilitates the development of single nucleotide polymorphic (SNP) markers which have shown to be a powerful genomic tool. However, their identification in polyploids is more difficult due to polyploid genome duplication (Clevenger et al., 2015). Until now SNPs for polyploidy asparagus have not been identified. In our case, the first step was to develop a data base for all accessions of the *ex situ* field collection with a set of SSR markers. The information obtained from this data base could be very useful to search for interspecific hybrids into the progenies from females of these accessions. In this study, a set of hybrid plants have been obtained in progenies derived from females of *A. persicus* and wild *A. officinalis* (AOK). The natural pollinations conducted to the obtention of the interspecific hybrids detected in this study may have been favored by the closely distance among the accessions in the field plot, around 2 m. In a previous study it was reported that asparagus male plants pollinate surrounding females up to a distance of 1.5 m (Huyskes, 1959). Different specific alleles were found in the diploid wild *A. officinalis* (AOK) accession, suggesting a greater genetic distance from the cultivated one. This accession shows thinner stem, and longer internodes than the naturalized *A. officinalis,* also showing a vining growth habit. In a previous study, a high genetic dissimilarity was obtained between this wild diploid *A. officinalis* accessions (AOK) compared to the naturalized AOD accession (Mousavizadeh et al., 2018).

In this study interspecific hybrids were not detected in the *A. verticillatus* progenies obtained from open pollination. On the other hand, interspecific hybrids with *A. verticillatus* were not detected in both *A. persicus* and *A. officinalis* (AOK) progenies. This result agrees with those reported by Ito et al. (2008) obtained in crosses between *A. officinalis* and *A. verticillatus*. The existence of cross genetic barriers between *A. officinalis* and *A. verticillatus* species might be explained by their huge genetic divergence (Fukuda et al., 2005; Ito et al., 2008; Kubota et al., 2012). Resistance to *Stemphilium vesicarium* Wallr. has been reported in *A. verticillatus* (Kanno and Yokoyama, 2011). More efforts should be made to overcome these barriers, for example looking for bridge species or increasing the number of crosses. In this sense, Falavigna et al. (2008) obtained infertility in a cross between *A. officinalis* and *A. acutifolius*, a distant phylogenetic asparagus subgenus species from the cultivated one. Nevertheless, a hybrid plant was obtained by these authors when an interspecific hybrid (*A. officinalis* × *A. maritimus*) was employed as genetic bridge after around two thousand crosses. With the aim to select the most suitable species to employ as genetic bridges, a good knowledge of the phylogenetic relationship between *Asparagus* spp. could be helpful. However, the *Asparagus* phylogeny remains largely incomplete so far.

Previous studies based on morphological traits (Mousavizadeh et al., 2015), number of rDNA 45S signals obtained by FISH analysis (Mousavizadeh et al., 2016) and SSR

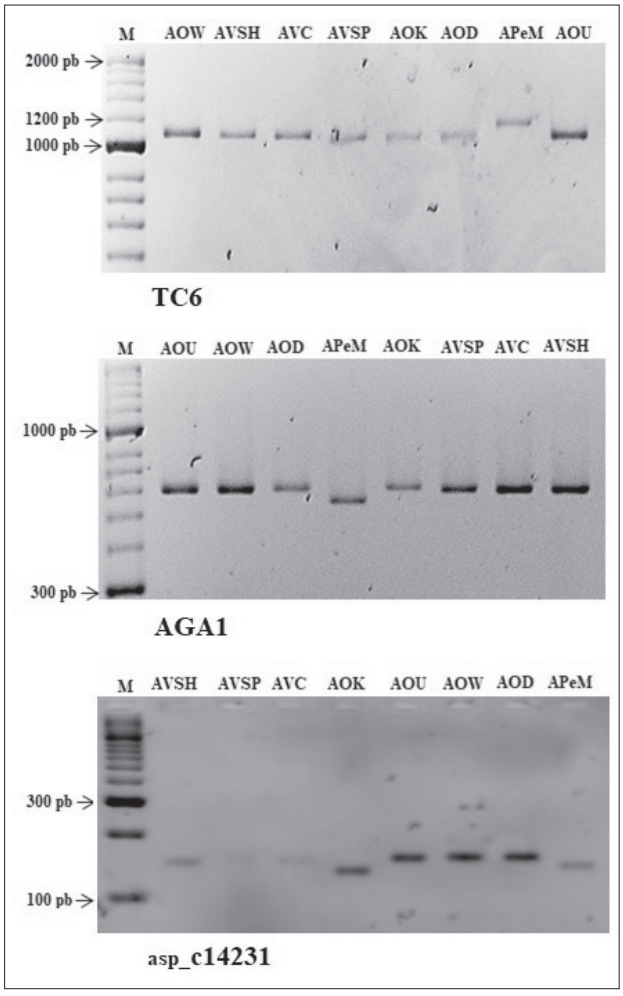

Figure 3. Agarose gels showing the amplification profile obtained with TC6, AGA1 and asp_c14231 SSR markers in different diploid *Asparagus* spp. accessions. Line M: DNA ladder Hyper Ladder TM 50 bp (Bioline, London, U.K.). AOW, AOK, AOD and AOU are *A. officinalis*; AVSH, AVC and AVSP are *A. verticillatus*; and APeM is *A. persicus*.

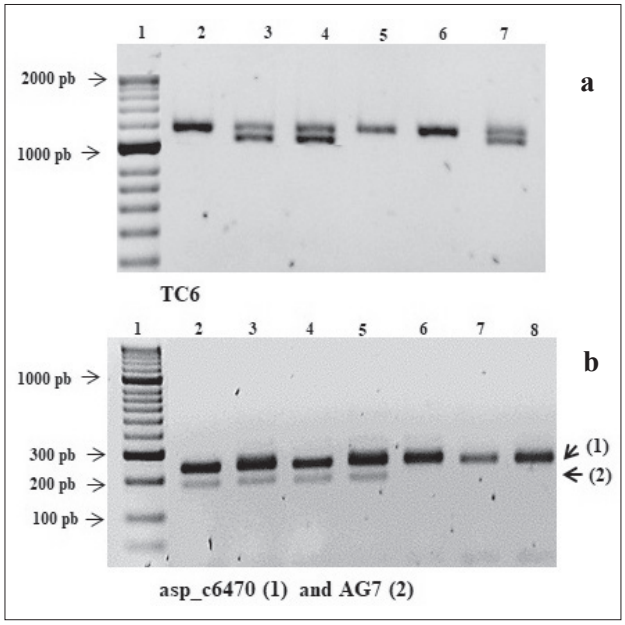

Figure 4. Amplification profile obtained with SSR markers of two different progenies: a) Progeny from *A. persicus* analyzed with TC6 marker. Lines 2, 5 and 6 only show the specific band of *A. persicus*, and lines 3, 4 and 7 are hybrid plants showing the specific band plus other band from other accession. b) Progeny from *A. officinalis* (AOK) analyzed by Multiplex PCR (AG7 and asp_c6470). Asp_c6470 (1) was used as positive control for absence in AG7 (2). Lines 6–8 show absence of amplicon for AG7 like *A. officinalis* (AOK) and lines 2–5 display hybrid plants with presence of amplicon present in other accessions. Line 1: DNA ladder Hyper Ladder TM 50 bp (Bioline, London, U.K.).

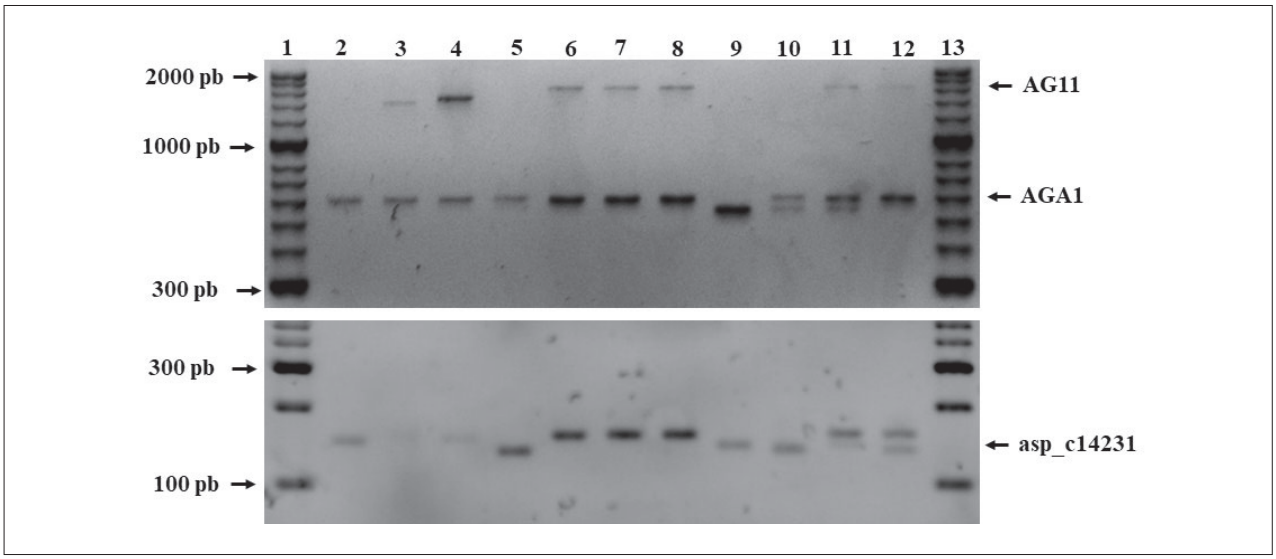

Figure 5. Agarose gel showing the amplification profile obtained with AG11, AGA1 and asp_c14231 SSR markers analyzed by two PCRs in different diploid *Asparagus* spp. Lines 1 and 13: DNA ladder Hyper Ladder TM 50 bp (Bioline, London, U.K.). Lines 2–4 are *A. verticillatus* (AVSH, AVSP and AVC). Lines 5–8 are *A. officinalis* (AOK, AOW, AOD and AOU). Line 9 is *A. persicus* (APeM). Lines 10–12 are hybrids: *A. persicus* × *A. officinalis* (AOK), *A. persicus* × *A. officinalis* (AOD, AOW or AOU) and *A. officinalis* (AOK) × *A. officinalis* (AOD, AOW or AOU).

markers (Mousavizadeh et al., 2021) have pointed out that *A. persicus* and *A. verticillatus* species seem to be more closely related compared to *A. officinalis*. However, the successful crossability found in this study between *A. persicus* and *A. officinalis* may indicate that these species are more closely related.

An experimental field trial plot established with different genetic resources and other distant phylogenetic *Asparagus* spp. from the cultivated species could be used to assess the obtention of new interspecific hybrids plants interesting in plant breeding. In these conditions, the possibilities to overcome cross genetic barriers between species might be enhanced because many pollinations per crosses by insects are expected avoiding the high time-consuming needed to carry out the hand pollinations. In this case the use of molecular markers along with the assessment of the ploidy level of progenies should be used as molecular tool to identify putative hybrids.

According to our results, SSR molecular markers have been a useful tool to validate the interspecific origin hybrid in handmade crosses and to detect hybrid plants in progenies derived from open pollination condition. Two new interspecific hybrids of cultivated asparagus with *A. persicus* and *A. breslerianus* as well as other intraspecific hybrids between cultivated and wild *A. officinalis* were obtained. Therefore, our results suggest that the hybrid plants detected in this study could contribute to enlarge the genetic base of this crop. With the aim to develop new asparagus germplasm, a backcrossing program toward cultivated plants has been recently initiated in our plant breeding program by employing all these hybrid plants with germplasm from *A. breslerianus*, *A. persicus* and wild *A. officinalis*.

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Authors' contributions

R.M. and J.G. conceived and designed the study; M.E.R., S.J.M. and R.M. developed the plant material; M.E.R. performed the DNA extraction and EST- SSR data obtention; M.E.R. and R.M. analyzed the EST-SSR data; M.E.R., J.G. and R.M. drafted the manuscript; S.J.M., Z.G. and T.B. 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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