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RESEARCH ARTICLE

Asparagus macrorrhizus Pedrol, Regalado et López-Encina, an endemic species from Spain in extreme extinction risk, is a valuable genetic resource for asparagus breeding

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Abstract *Asparagus maritimus* is a species distributed in sandy soils along the Mediterranean coast. It has been reported as salt tolerant and resistant to rust. The wild asparagus species are a very important genetic resources for asparagus breeding because the current commercial cultivars have a narrow genetic base. Until recently, the only population of *A. maritimus* catalogued in Spain was a small population, which is at high extinction risk, located around the coastal lagoon "Mar Menor" in the region of Murcia. Different studies carried out in the current work support the recent description of this population as a new species named *Asparagus macrorrhizus*. Plants from three populations of *A. maritimus* were used to carry out studies of characterization and the results were compared with plants of *A. macrorrhizus*. The morphological studies showed clear differences between the populations of *A. maritimus* and *A. macrorrhizus*. One of the differences found between these populations was at the ploidy level. The plants of *A. maritimus* were hexaploid (2n = 6x = 60), while the plants of *A. macrorrhizus* were dodecaploid (2n = 12x = 120). Also, the flavonoid composition showed that *A. maritimus* contains six different flavonoids while in *A. macrorrhizus* 90 % of the flavonoid content corresponds to only one flavonoid (Nicotiflorin) followed by minor quantities of other two. Another difference between these populations was supported by the principal coordinate analysis (PCoA) using data from 4 EST-SSRs markers amplified in plants of *A. maritimus* and *A. macrorrhizus*, and clearly separates the two species. The differences

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found in this work highlight the importance of *A. macrorrhizus* as a possible genetic resource for asparagus breeding. The distribution of *A. macrorrhizus* is limited to the area surrounding the "Mar Menor" lagoon. The prospections carried out in the last years indicated the high risk of extinction of this species due to the urbanization of this natural habitat. Therefore, we have included *A. macrorrhizus* in our germplasm bank in vivo and in vitro as well as in the breeding programs.

Keywords Asparagus breeding · Endangered species · Flavonoid · Genetic resources · Morphological characterization · Ploidy level · Principal coordinates analysis

Introduction

Asparagus L. is a large genus widespread in temperate and tropical regions of Africa, Asia, Australia and Europe (Kubota et al.2012). The genus comprises three subgenera: *Asparagus*, *Protasparagus* and *Myrsiphyllum* (Clifford and Conran 1987). The species of subgenus *Asparagus* are dioecious, with unisexual flowers, whereas the species belonging to *Protasparagus* and *Myrsiphyllum* subgenera are hermaphroditic. The basic chromosome number of the Asparagus genus is ten ($x = 10$) and polyploidization seems to be common in this genus. Thus, polyploid species with different ploidy levels have been described in some *Asparagus* spp. (Valdes 1980; Xinqi and Tamanian 2000; Kanno and Yokoyama 2011). This fact suggests that polyploidization may have played an important role in the evolution of Asparagus (Castro et al. 2013).

Garden asparagus (*Asparagus officinalis* L.) is the most economically important species of the genus and is cultivated worldwide as a vegetable. Nevertheless, most of the commercial varieties derive from the old cultivar 'Purple Dutch' (Ellison 1986; Geoffriau et al. 1992) and, so, the asparagus genetic base is extremely narrow (Brettin and Sink 1992; Geoffriau et al. 1992; Lallemand et al. 1994; Khandka et al. 1996; Moreno et al. 2006). Therefore, the introgression of agronomically important traits from other Asparagus species would be valuable for breeding programs in order to increase the genetic pool of cultivated *A. officinalis*. For example, the introgression of *A. maritimus* into *A. officinalis* could be interesting for asparagus breeding due to the high level of salt tolerance and the resistance to rust showed by *A. maritimus* (Venezia et al. 1993; Alberti et al. 2004).

A. maritimus (L.) Miller is a wild species distributed on sandy soils along the Mediterranean coast (Bozzini 1959; Valdes 1980). Phylogeny studies employing molecular markers showed a close relationship between *A. maritimus* and *A. officinalis* (Fukuda et al. 2005; Kubota et al. 2012). This fact may have allowed the development of interspecific hybrids between both species (Falavigna et al. 2008) and could explain the interspecific origin (*A. maritimus* × *A. officinalis*) proposed to the Spanish tetraploid asparagus landrace "Morado de Huétor" (Moreno et al. 2008), one of the few current asparagus varieties of asparagus whose origin is not 'Purple Dutch'. In Spain, there is only a small population of *A. maritimus* growing around the "Mar Menor", a coastal lagoon in the region of Murcia (Sánchez-Gómez et al. 2002). Nowadays, this population is protected by the regional government with the "Special Interest" category due to its high extinction risk (Sánchez-Gómez et al. 2007). In 2008, the estimated number of plants of this population was 694, distributed in three subpopulations, and the species was included in the category "Critically Endangered" in the Red Book of Spanish Vascular Flora 2008 (Sánchez-Gómez et al. 2008). The main problem to preserve these three unique subpopulations is the high urbanistic pressure caused by the touristic character of the "Manga del Mar Menor" area. In the last 50 years more than 90 % of the potential habitat of this species has been constructed, endangering the survival of this population. Another problem is the dioecious character of *A. maritimus*. The reduced number of female plants increases the difficulty of the reproduction and preservation of this species generally distributed in small subpopulations (Sánchez-Gómez et al. 2007). Previous studies employing molecular markers in several *A. maritimus* populations pointed out the singularity of the "Mar Menor" population and a different evolutionary pathway has been suggested for this population (Moreno et al. 2008; Castro et al. 2013). Recently, Pedrol et al. (2013) catalogued the Spanish population of *A. maritimus* as a new species within the subgenus *Asparagus* and named it *Asparagus macrorrhizus* Pedrol, Regalado et López-Encina.

The objective of this work is the study of the differences between this new species, A. *macrorrhizus*, and different populations of *A. maritimus*. Morphological studies, ploidy analysis, phytochemical composition studies (flavonoid characterization), phylogenetic analyses and geographical distribution studies were performed to show the important differences found in *A. macrorrhizus* that confirm this species as a valuable genetic resource for asparagus breeding.

Materials and methods

Plant material

Plants of *A. macrorrhizus* from the population from the "Manga del Mar Menor" and plants of *A. maritimus* from two different populations from Albania and Padova (Italy) were used in the assays performed in this work. The plant number used in each study is indicated in the corresponding section of materials and methods. In some studies, *A. officinalis* and a population of *A. maritimus* from Venice (Italy) were included as external control (Table 1).

Morphological study

The morphological analysis was separately performed in the aerial part of the plants and the rhizome-root system or subterranean part of the plant. The same three plants of each accession were used in both morphological analyses. The low number of plants studied was due to that morphological analysis of the rhizome-root system supposes the destruction of the plant studied, and we try to avoid the destruction of plants of a species with high risk of extinction such as *A. macrorrhizus*.

To study the aerial part, a total of 17 aerial organs were measured in plants of *A. macrorrhizus* and *A. maritimus* from Albania and Padova (Table 2). *A. maritimus* from Venice was not characterized in this morphological study because there were not plants of this population grown under the same conditions as the other three populations. For each plant, a minimum of five measurements per organ were scored. The mean value for each accession was calculated for each measurement. A variance analysis (ANOVA) was employed to detect significant differences between the mean values of the accessions for each trait. In the cases where the measurements had a large variation (for instance, the length of the primary branches), the interval of maximum and minimum average values for the three different samples were presented instead of the mean.

The morphological study of the rhizome was more complicated due to the subterranean nature of this part of the plant and the impossibility of digging up the rhizome-root system completely. Even so, we were able to dig up plants of *A. macrorrhizus* and *A. maritimus*. To analyze the structure and distributions of the primary roots and the groups of rhizome buds in each species, sand and secondary roots were eliminated after digging up the plants.

Ploidy analysis

The ploidy level of *A. macrorrhizus* and *A. maritimus* from Albania, Padova and Venice was determined by estimating the relative DNA content using flow cytometry (Ploidy Analyser PA-I; Partec GmbH, Münster, Germany) of ten plants belonging to each population. 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

Table 1 Characteristics of the asparagus accessions used in different studies: (A) morphological study; (B) ploidy level analysis; (C) flavonoid characterization

Species	Ploidy level	Origin	Donor	Use
A. macrorrhizus	12x	Cartagena (Spain)	Dr. JJ Martinez	ABC
A. maritimus	6x	Albania	Lecce Botanical Garden (Italy)	ABC
A. maritimus	6x	Padova (Italy)	Botanic Garden of Padova	ABC
A. maritimus	6x	Venice (Italy)	Dr. Falavigna	BC
A. officinalis L. 'Grande'	2x	California Seeds (USA)	–	BC

Table 2 Morphological study of *Asparagus maritimus* from Padova and Albania and *Asparagus macrorrhizus*

Morphological trait	*Asparagus maritimus*		*Asparagus macrorrhizus*
	Padova (Italy)	Albania	
Plant height (cm)	80.67 ± 11.89a	83.00 ± 8.39a	51.53 ± 12.37b
Plant height to first panicle branch (cm)	10.50 ± 1.38a	11.00 ± 1.58a	8.13 ± 2.88a
Number of primary branches	35.50 ± 4.18b	40.50 ± 3.62a	22.7 ± 2.18c
Number of secondary branches	8.00 ± 2.83b	11.83 ± 1.47a	NED
Internode length of primary branches (cm)	2.67 ± 0.41a	3.25 ± 0.69a	1.87 ± 0.47b
Internode length of secondary branches (cm)	2.08 ± 0.20a	1.88 ± 0.51a	NED
Diameter of main stem (mm)	5.17 ± 0.75b	6.67 ± 1.03a	3.53 ± 0.52c
Diameter of primary branches (mm)	2.17 ± 0.41a	2.33 ± 0.52a	1.93 ± 0.18a
Diameter of secondary branches (mm)	1.08 ± 0.20a	1.08 ± 0.20a	NED
Diameter of leaves (mm)	1.00 ± 0.00a	1.00 ± 0.00a	1.23 ± 0.42a
Length of primary branches (minimum–maximum cm)	3.5–35	2.5–44	2.0–19
Length of secondary branches (minimum–maximum cm)	1.5–11	2.0–19	NED
Length of leaves (minimum–maximum cm)	0.6–2.3	0.3–2.7	0.6–3.4
Number of scale under the lowest panicle branch	5.17 ± 0.75a	5.83 ± 0.75a	5.13 ± 0.92a
Length of spears scales (mm)	8.67 ± 1.21b	10.33 ± 0.82a	6.93 ± 1.16c
Width of spears scales (mm)	4.83 ± 0.41a	5.33 ± 0.82a	3.53 ± 0.74b
Number of cladodes	5.50 ± 1.05a	5.50 ± 0.55a	7.27 ± 2.19a

Means (±SE) followed by the same letter within rows do not differ according to Tukey pairwise comparison test at level $P = 0.05$

NED No existing data; plants of *Asparagus macrorrhizus* did not show secondary branches

PartecCyStain 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 PartecCyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer, about 1.6 mL). Finally, the samples were analyzed after 30 s of incubation. *A. officinalis* 'Grande' (2n = 2x = 20) was used as an external standard.

Principal coordinate analysis (PCoA)

A PCoA was performed on a binary matrix (0 and 1) using GENALEX 6 software (Peakall and Smouse 2006). The binary matrix was previously developed by Castro et al. (2013) in a cluster study carried out with four EST-SSRs markers amplified in plants belonging to three accessions of *A. maritimus* and plants of *A. macrorrhizus*. These three accessions of *A. maritimus* correspond to the three populations of this species (Albania, Venice, Padova) used in the others studies of this work. Nine to ten plants per accessions were analyzed. In the aforementioned binary matrix, the alleles were scored as present (1) or absent (0) for each marker.

Flavonoids characterization

The spears were harvested from experimental fields under controlled conditions, cut to the same length, and kept under refrigerated conditions (4 °C) from the field to the laboratory to minimize the environmental influence and the storage conditions on the flavonoid profile. Prior to being analyzed, the spears were washed with sodium hypochlorite solution (50 ppm of chlorine active) and cut to a distance of 20 cm from the tip. Asparagus samples were weighed, frozen at −20 °C and freeze-dried. The plant tissue was ground into a fine powder and stored at −20 °C for further analysis. Spears of ten plants of each population studied (Table 1) were characterized.

The flavonoids characterization was made by following the methodology developed by Fuentes-Alventosa et al. (2007, 2008). Authentic standards of quercetin (Q), kaempferol (K), isorhamnetin (IR) and

rutin (quercetin 3-*O*-rutinoside) were purchased from Sigma-Aldrich Quimica (Madrid, Spain); kaempferol-3-*O*-rutinoside (nicotiflorin), isorhamnetin 3-*O*-rutinoside (narcisin) and isorhamnetin 3-*O*-glucoside were purchased from Extrasynthese (Genay, France). All solvents were of HPLC grade purity (Romyl, Teknokroma, Barcelona, Spain). All sample solutions were prepared using Milli-Q water. For flavonoids extraction, the different samples consisting of 2.5 g of freeze-dried material were extracted with 100 mL of 80 % ethanol (EtOH). The samples were blended in a Sorvall Omnimixer, model 17106 (DuPont Co., Newtown, CT), at maximum speed for 1 min and then filtered through filter paper. Ethanolic extracts were stored at −20 °C until analysis by HPLC. All extractions were made in triplicate.

The analyses of flavonoids were carried out using a Jasco-LC-Net II ADC liquid chromatograph system equipped with a diode array detector (DAD). Flavonoid compounds were separated by using a SYNERGI 4 μHYDRO-RP80A reverse phase column (25 cm × 4.6 mm i.d., 4 μm particle size; Phenomenex, Macclesfield, Cheshire, U.K.). The gradient profile for the separation of flavonoids was formed using solvent A [10 % (v/v) aqueous acetonitrile plus 2 mLL^{-1} acetic acid] and solvent B (40 % methanol, 40 % acetonitrile, 20 % water plus 2 mLL^{-1}acetic acid) in the following program: the proportion of B was increased from 10 to 42.5 % B for the first 17 min, then to 70 % B over the next 6 min, maintained at 70 % B for 3.5 min, then to 100 % B over the next 5 min, maintained at 100 % B for 5 min and finally returned to the initial conditions. The flow rate was 1 mL min^{-1} and the column temperature was set at 30 °C. Spectra from all peaks were recorded in the 200–600 nm range and the chromatograms were acquired at 360 nm.

The identification of individual flavonoid glycosides was carried out using their retention times. Both spectroscopic and mass spectrometric data, and the quantification of individual flavonoid glycosides was directly performed by HPLC–DAD using an eight-point regression curve in the range of 0–250 μg on the basis of standards. Monoglycosides and diglycosides standards were purchased from Extrasynthese and triglycosides were obtained from Phytochemicals and Food Quality Group (Instituto de la Grasa, CSIC) who had previously isolated and purified them from samples of *A. officinalis* (Fuentes-Alventosa et al. 2008). Results were calculated from the mean of three replicates. Comparisons among samples were performed by the ANOVA test and the LSD method at 95 % confidence level.

Geographical distribution of *Asparagus macrorrhizus*

The data showed in this work were obtained from prospecting conducted by environmental agents and biologists from the Directorate General for the Environment of the government of the region of Murcia from 2004 to 2014. The different individuals were placed by a receptor GPS model Etrex Legend of Garmin, without later corrections, with coordinates ETRS 1989 UTM ZONE 30N. The maps were prepared with the software ArcMap 10.1 of ArcGis, coordinates ETES 1989 UTM Zone 30N, using as base map a digital terrain model of 25 m resolution (MDT25-LIDAR, Instituto Geográfico Nacional de España) and an administrative information layer downloaded from http://www.gadm.org/. The first map corresponds to the data collected in 2007 and the second one corresponds to the data collected from 2012 to 2014, within the Conservation Program of the Protected Wild Flora of the government of the region of Murcia.

Results

Morphological study

Means and standard errors of the studied traits are presented in Table 2. The differences between the two accessions of *A. maritimus* from Padova (Italy) and Albania were very small compared to the differences between these two accessions and *A. macrorrhizus*. Both *A. maritimus* populations showed significant differences ($P = 0.05$) in the number of primary and secondary branches, the diameter of main stem and the length of spear scales (Table 2). *A. macrorrhizus* showed differences respect with the two accessions of *A. maritimus* in most of the traits studied. It should be highlighted that the plants of *A. macrorrhizus* did not present secondary branches, the number of primary branches was lower than the number recorded in *A. maritimus* and showed a smaller size in general. It was significantly shorter in total plant height, internodes

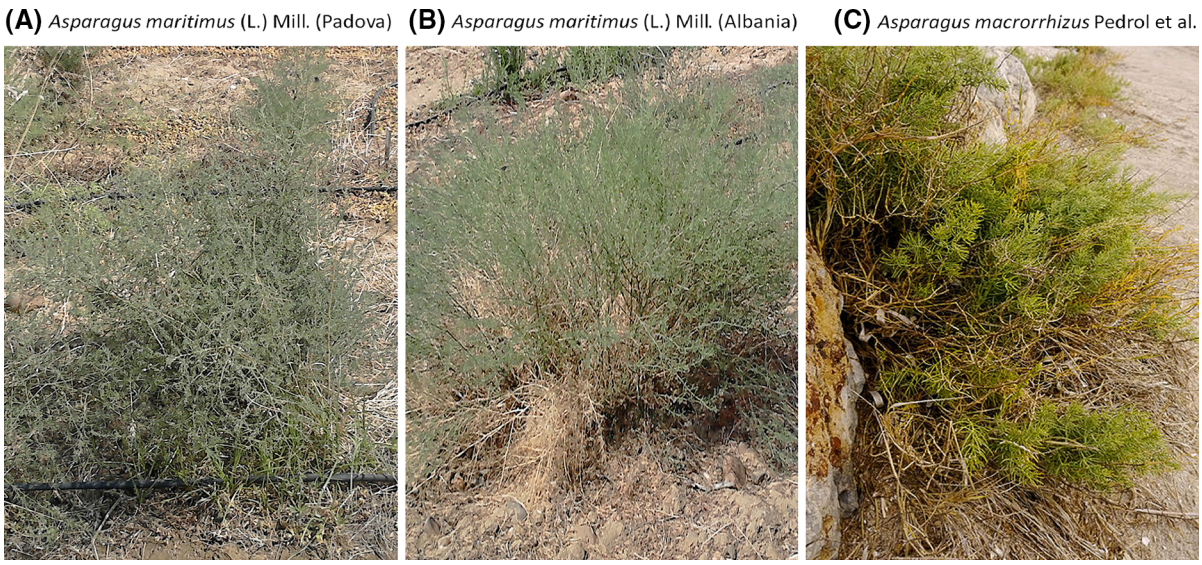

Fig. 1 Accessions used in the morphological study: **A** *Asparagus maritimus* (Padova), **B** *Asparagus maritimus* (Albania), **C** *Asparagus macrorrhizus*

length of primary branches and diameter of main stem. Figure 1 shows a plant of each accession growing in experimental fields or in nature.

Clear morphological differences among the aerial parts of these plant specimens belonging to the species *A. maritimus* and *A. macrorrhizus* were observed (Fig. 2A). The rhizome of both species presented differences in their structure (Fig. 2B, C). In the case of *A. maritimus*, the rhizome shows a circular and centrifugal growth pattern, with the rhizome buds concentrated in circular clusters (Fig. 2F) and all roots developing from these points. However, the architecture of *A. macrorrhizus* rhizome is different. It is characterized by the presence of transversely lengthened roots. The rhizome buds are scattered throughout these enlarged roots (Fig. 2G) and the root development follow an identical pattern.

Ploidy analysis

A. officinalis 'Grande' ($2x = 20$) was used as an external diploid standard in all ploidy level analysis, establishing a value of 25 to the peak corresponding to the G1 somatic nuclei ($2n = 2x = 20$) and a value of 50 to the peak corresponding to the G2 somatic nuclei ($4n = 4x = 40$) in the flow cytometry histogram (Fig. 3A). The flow cytometry histogram of the three accessions of *A. maritimus* (from Albania, Venice and Padova) showed the same two peaks (Fig. 3B–D). The main peak situated in a value of 75 represents G1 somatic nuclei ($2n = 6x = 60$) and the secondary peak located in a value of 150 represents G2 somatic nuclei ($4n = 12x = 120$), indicating that these plants are hexaploid ($6x = 60$). The flow cytometry histogram of *A. macrorrhizus* showed only one peak (Fig. 3E) situated in a value of 150 that represents G1 somatic nuclei ($2n = 12x = 120$). The secondary peak corresponding to the G2 somatic nuclei ($4n = 24x = 240$) should appear in a value of 300 but this value is outside the range of the cytometry. These results indicate that *A. macrorrhizus* is dodecaploid (12x).

Principal coordinate analysis (PCoA)

The PCoA results showed that *A. macrorrhizus* was clearly separated by the first component from the *A. maritimus* populations whereas these ones were separated between them, with some overlap, according to the second component (Fig. 4). The variability showed by the nine plants of *A. macrorrhizus* seems to be larger than the obtained in each one of the three populations of *A. maritimus* according their distribution on the two components.

Flavonoid characterization

The flavonoid profiles of *A. macrorrhizus* and *A. maritimus* have a different composition. Only three

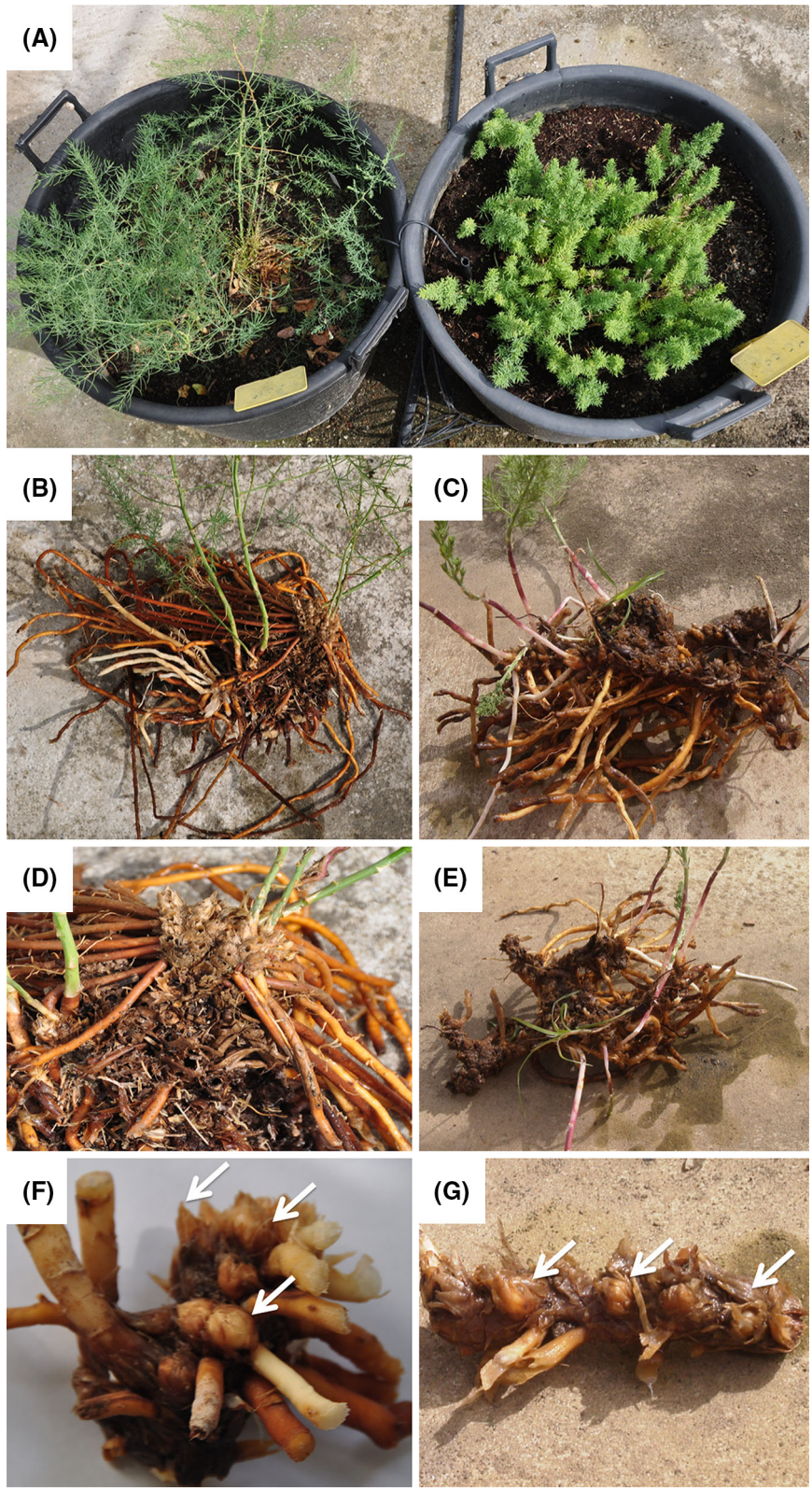

Fig. 2 **A** Plants of *Asparagus maritimus* (*left*) and *Asparagus macrorrhizus* (*right*) growing in pots in a greenhouse at the IHSM La Mayora. **B** Rhizome of *Asparagus maritimus.* **C** Rhizome of *Asparagus macrorrhizus*. **D** Detail of the rhizome of *Asparagus maritimus*. **E** Detail of the rhizome of *Asparagus macrorrhizus*. **F** Rhizome buds of *Asparagus maritimus*. **G** Rhizome buds of *Asparagus macrorrhizus*. *Arrows* indicate rhizome buds

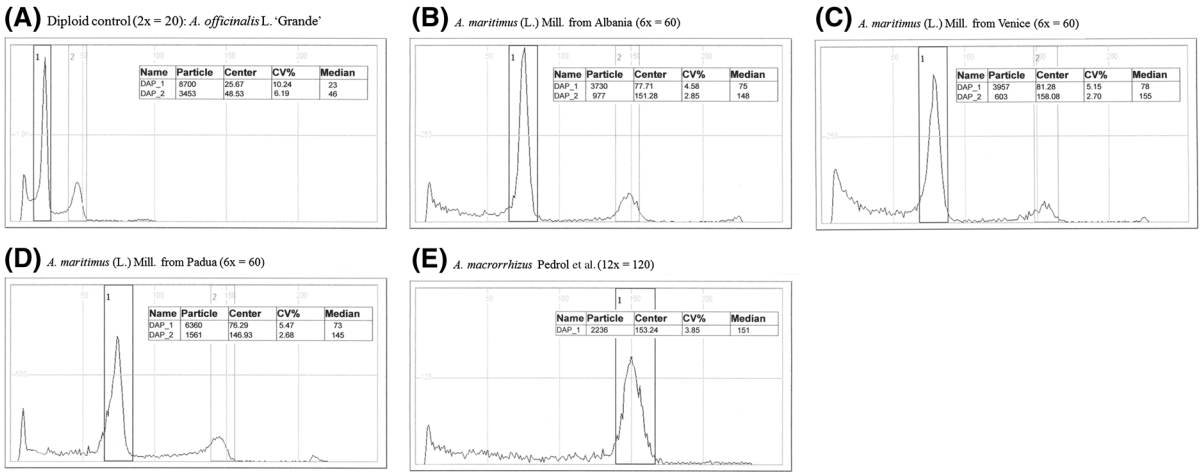

Fig. 3 Flow cytometry histograms. **A** Diploid control (2x = 20) *A. officinalis* 'Grande'. **B** *A. maritimus* from Albania (2n = 6x = 60). **C** *A. maritimus* from Venice (2n = 6x = 60). **D** *A. maritimus* from Padova (2n = 6x = 60). **E** *A. macrorrhizus* (2n = 12x = 120)

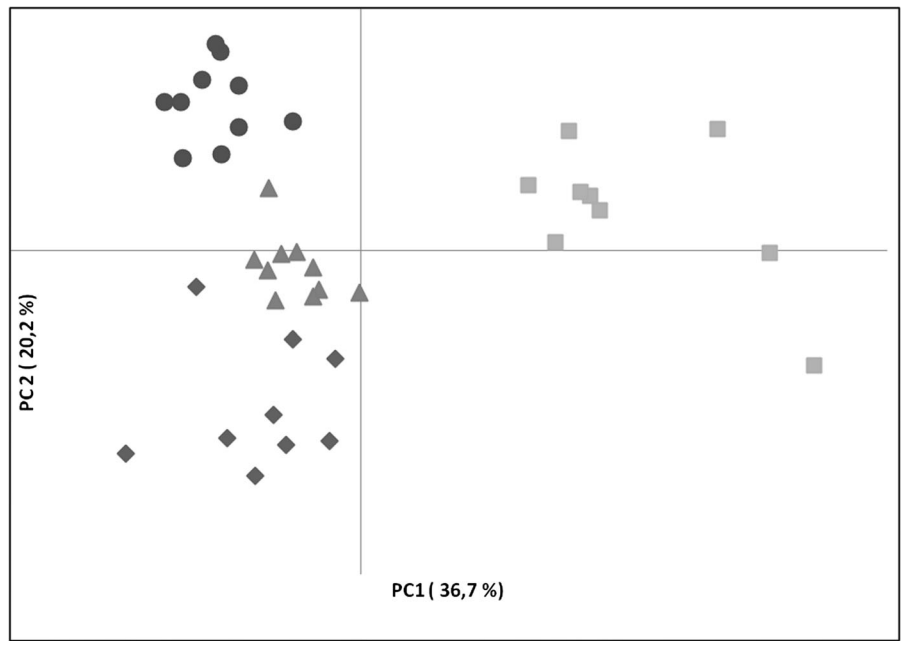

Fig. 4 Principal coordinate analysis based on profiles from EST-SSR markers analyzed in asparagus samples consisted of *A. maritimus* from Padova (*circle*), *A. maritimus* from Venice (*triangle*), *A. maritimus* from Albania (*diamond*) and *A. macrorrhizus* (*square*)

flavonoids were detected in *A. macrorrhizus* and more than 90 % of the flavonoid content corresponded to Nicotiflorin (kaempferol-rutinoside). Minor quantities of narcisine (Isorhamnetin-rutinoside) and rutin were also observed (Table 3; Fig. 5). Six flavonoids gyclosides (derived from 3 aglycones, quercetin, kaempferol e isorhamnetin) were detected in the three populations of *A. maritimus* studied in this work (Fig. 5B–D), although there were differences in the percentage among them. *A. officinalis* cv. Grande was included as control. It has a profile consisting of a major peak, which corresponds to rutin and represented more than 70 % of the total flavonoids. Other compound identified as flavonoid glycosides from Quercetin was also present but in a lower percentage.

Table 3 Flavonoid content in relative percentage (%) and mg kg^{-1} fresh (in parenthesis) in three populations of *A. maritimus* from Albania, Venice and Padova and a population of *A. macrorrhizus*

Accessions	*Q-triglyc*	Q-triglyc	IR-triglyc	Rutin	IR-triglyc	Nicotiflorin	Narcisin	Total (mg kg^{-1} fresh)
A. maritimus (Albania)	22 ± 0 %^{a} (146 ± 7^{a})	15 ± 0 %^{c} (99 ± 6^{b})	10 ± 0 %^{b} (64 ± 1^{a})	37 ± 1 %^{a} (247 ± 16^{a})	3 ± 0 %^{a} (23 ± 1^{a})	6 ± 0 %^{d} (41 ± 0^{d})	6 ± 0 %^{b} (38 ± 1^{a})	659 ± 29^{a}
A. maritimus (Venice)	22 ± 3 %^{a} (68 ± 10^{b})	19 ± 1 %^{b} (61 ± 1^{c})	12 ± 1 %^{a} (39 ± 2^{b})	21 ± 1 %^{c} (66 ± 0^{c})	0 ± 0 %^{b} (0 ± 0^{b})	17 ± 0 %^{c} (53 ± 2^{c})	9 ± 0 %^{a} (29 ± 0^{b})	315 ± 9^{d}
A. maritimus (Padova)	15 ± 0 %^{b} (59 ± 0^{c})	30 ± 0 %^{a} (119 ± 4^{a})	6 ± 1 %^{c} (24 ± 3^{c})	26 ± 2 %^{b} (104 ± 11^{b})	0 ± 0 %^{b} (0 ± 0^{b})	21 ± 1 %^{b} (82 ± 1^{b})	3 ± 0 %^{c} (12 ± 1^{c})	400 ± 10^{c}
A. macrorrhizus	0 ± 0 %^{c} (0 ± 0^{d})	0 ± 0 %^{d} (0 ± 0^{d})	0 ± 0 %^{d} (0 ± 0^{d})	5 ± 0 %^{d} (21 ± 0^{d})	0 ± 0 %^{b} (0 ± 0^{b})	92 ± 0 %^{a} (408 ± 11^{a})	3 ± 0 %^{c} (13 ± 1^{c})	442 ± 12^{b}

Different letters indicate significant differences between values according to ANOVA test and LSD method at 95 % confidence level

Geographical distribution of *Asparagus macrorrhizus*

The data provided by the first prospecting, conducted in 2007 (Fig. 6A), indicated the existence of at least twenty points with the presence of more than ten plants of this species. Most of these points are concentrated at the end of the "Manga del Mar Menor", in the area called "La Veneziola". In the same prospecting, twenty-three points with five to ten plants and eighty-four points with less than five plants were detected. These points were scattered along the "Manga del Mar Menor" but most of them were also concentrated in "La Veneziola". According to the second prospecting carried out from 2012 to 2014, the situation has drastically changed (Fig. 6B). Only one point with more than ten plants was detected in "La Veneziola". In the same area three points with five to ten plants were detected. Other forty-one points with less than five plants completed the data of this prospecting. Only nine of these points were detected outside "La Veneziola". In summary, there has been a severe decrease in the number of points with presence of *A. macrorrhizus* and the number of plants in each of these points along the "Manga del Mar Menor" in the last 7 years, especially in the area with higher presence of this species: "La Veneziola".

Discussion

The genus *Asparagus* includes more than 200 species (Dahlgren et al. 1985), although this number is subjected to change. Nowadays, the discovery and description of new species within the genus *Asparagus* still continues. In the last years, new species of *Asparagus* have been described. Burrows and Burrows (2008) described three new species of *Asparagus* from South Africa. Demissew (2008) reported four new species from the *Zambezi River basin*, and two new species from Iran were described by Hamdi and Assadi (2009, 2012). In addition, the taxon *Asparagus prostratus,* which previously was considered as a subspecies within *A. officinalis*, has been considered to a new species (Kay et al. 2001). However, only the species of the subgenera *Asparagus* are compatible with *A. officinalis*, making impossible the development of hybrids between *A. officinalis* and species of the subgenus *Protasparagus* and *Myrsiphyllum*

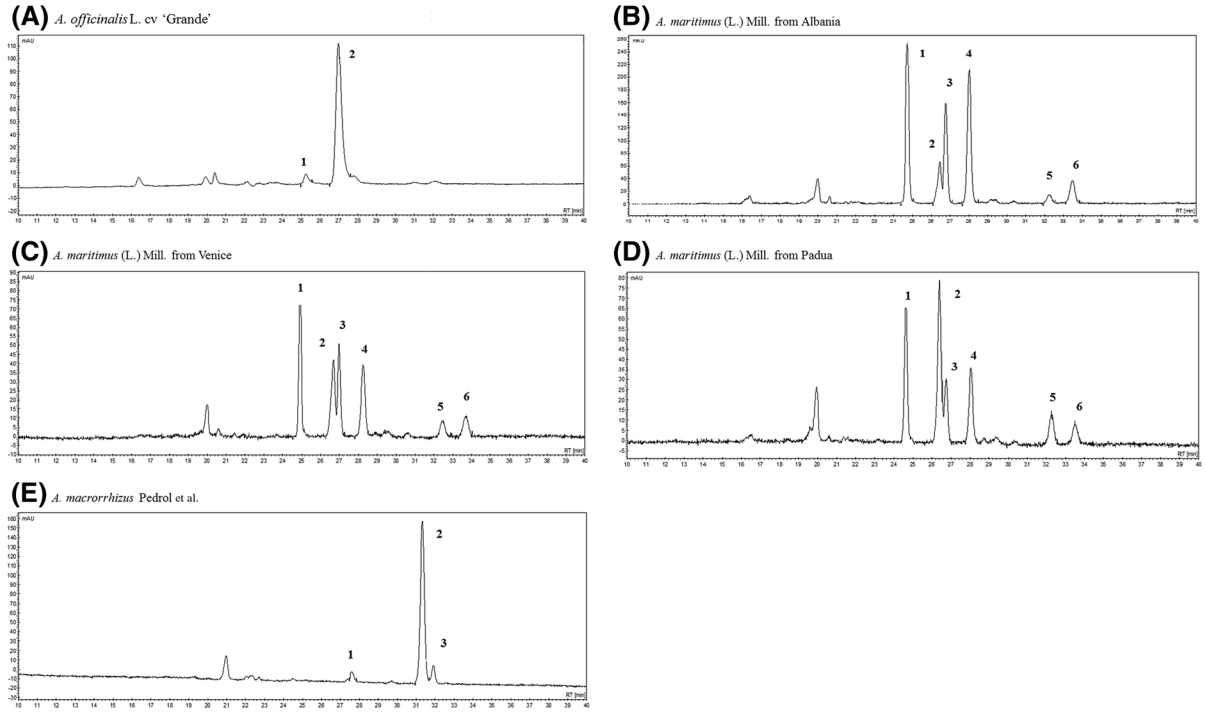

Fig. 5 HPLC elution profile of different species of *Asparagus sp*. with detection at 360 nm. **a** *A. officinalis* ('Grande'). Key to peak identity: *1* quercetin-triglycoside; *2* rutin. **b** *A. maritimus* from Albania. Key to peak identity: *1* quercetin-triglycoside; *2* quercetin-triglycoside; *3* isorhamnetin-triglycoside; *4* rutin; *5* nicotiflorin; *6* narcisin. **c** *A. maritimus* from Venice. Key to peak identity: *1* quercetin-triglycoside; *2* quercetin-triglycoside; *3* Isorhamnetin-triglycoside; *4* rutin; *5* nicotiflorin; *6* narcisin. **d** *A. maritimus* from Padova Key to peak identity: *1* quercetin-triglycoside; *2* quercetin-triglycoside; *3* isorhamnetin-triglycoside; *4* rutin; *5* nicotiflorin; *6* Narcisin. **e** *A. macrorrhizus* with detection at 360 nm. Key to peak identity: *1* rutin; *2* nicotiflorin; *3* narcisin

(Kunitake et al. 1996; Ito et al. 2008; Kubota et al. 2012). This incompatibility means that the number of wild species that can be used to increase the genetic pool of *A. officinalis* is very limited and, therefore, the high value as genetic resource of new species of the subgenus *Asparagus* as *A. macrorrhizus*.

Before the description of *Asparagus macrorrhizus* as a new species within the subgenus *Asparagus* (Pedrol et al. 2013), the plants of this species were considered as a population of *A. maritimus* with evolutionary significant differences (Sánchez-Gómez et al. 2007). The studies presented in this work support the cataloging of this population as a new species and show the important differences between *A. macrorrhizus* and the different populations of *A. maritimus*, which make *A. macrorrhizus* an important genetic resource.

Different authors have investigated the relationships within *Asparagus* genus employing different DNA studies approach, such as restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA or ribosomal DNA (Lee et al. 1997; Štajner et al. 2002; Moreno et al. 2008), sequence polymorphisms of the non-coding chloroplast DNA or ribosomal DNA regions (Fukuda et al. 2005; Kubota et al. 2012; Castro et al. 2013) and molecular markers obtained from expressed sequence tag-derived simple sequence repeat (EST-SSR) (Castro et al. 2013).*Asparagus maritimus* is one of the species included in these studies and was placed close to *A. officinalis* regardless of the origin of the population evaluated. In the aforementioned studies, most of the *A. maritimus* populations evaluated were grouped together in the different cluster analysis carried out (Štajner et al. 2002; Moreno et al. 2008; Castro et al. 2013). One exception was the Spanish population of *A. maritimus*, recently catalogued as *A. macrorrhizus* (Pedrol et al. 2013). This population was clearly separated from the other *A. maritimus* populations in the two cluster analysis developed using molecular markers (Moreno

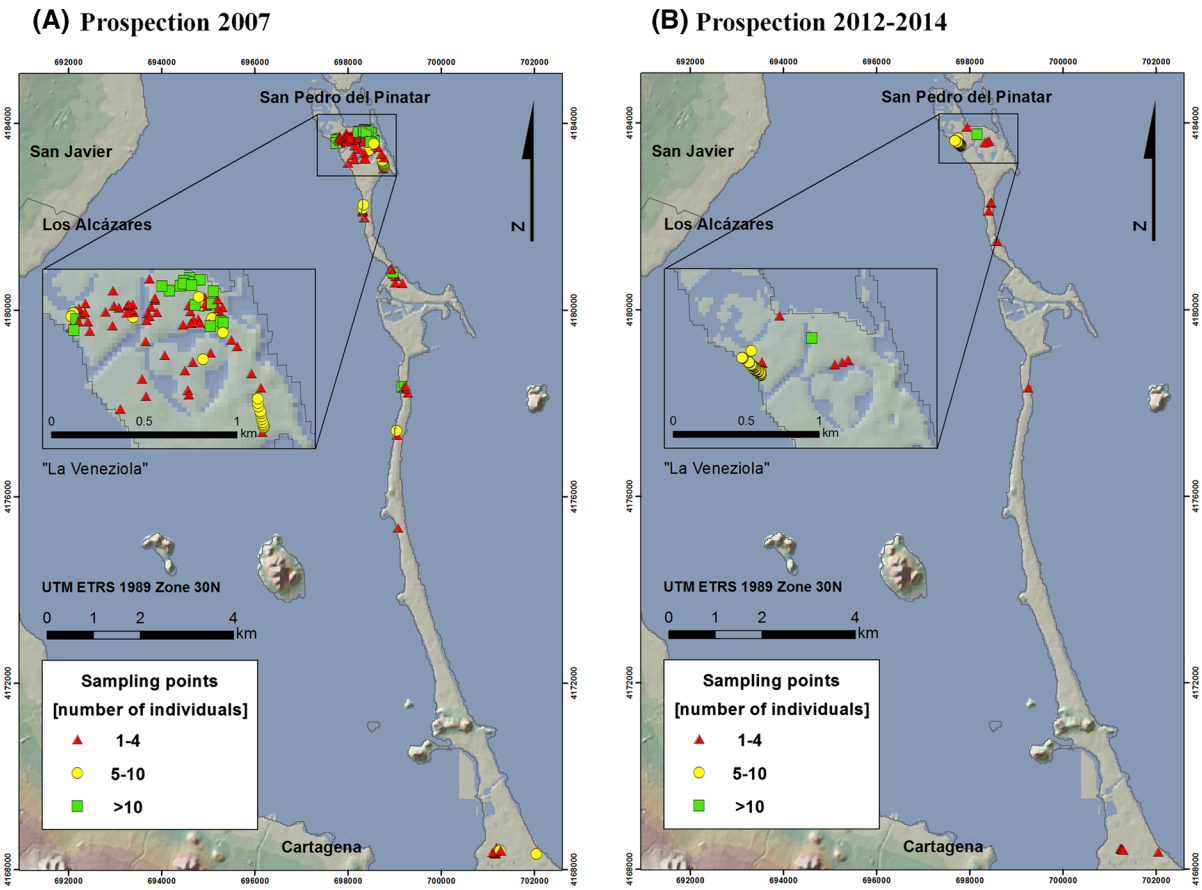

Fig. 6 Geographical distribution of *Asparagus macrorrhizus*. **A** Data collected in 2007 prospecting within the Conservation Program of the Protected Wild Flora of the government of the region of Murcia. **B** Data collected in 2012–2014 prospecting within the same Conservation Program

et al. 2008; Castro et al. 2013). In the current study, the result obtained using Principal Coordinates Analysis agrees with the previous studies and supports that the Spanish population do not belong to *A. maritimus* species.

The differences between *A. macrorrhizus* and *A. maritimus* go beyond the molecular differences, presenting other differences such as the morphology of the aerial part of the plants and the rhizome architecture, the ploidy level or the flavonoid composition. The morphology of the aerial part of *A. macrorrhizus* clearly differs from the two populations of *A. maritimus* studied (Fig. 1; Table 2). The most notorious difference was the absence of secondary branches in *A. macrorrhizus* while the plants of *A. maritimus* showed from 8 to 12 secondary branches, otherwise the differences found between the two populations of *A. maritimus* were slight. The most significant morphological difference of *A. macrorrhizus* respect to *A. maritimus* was found in their subterranean part or rhizome-root system (Fig. 2). The structure of the rhizome-root system in *A. maritimus* consists in a pattern of circular rhizome bud clusters with the roots developing from these points and is similar to the structure described in other closely related species such *A. officinalis*, *A. pseudoscaber* Grec. and *A. brachyphyllus* Turz. (Carmona-Martin et al. 2014; Regalado et al. 2015). However, *A. macrorrhizus* shows a rhizome-root system very different respect to these species. The rhizome bud clusters and the points of growth of roots are scattered throughout transversely extended rhizomes (Fig. 2g).

Different ploidy levels have been described in *A. maritimus*. Bozzini (1959) proposed a tetraploid ploidy level for *A. maritimus* studying one population from Venice (Italy) and plants from four different

botanical gardens. More recently hexaploid populations have been described regardless of their origin (Slovenia, Albania or Italy) (Štajner et al. 2002; Moreno et al. 2008; Castro et al. 2013). However, the Spanish population of this species, now catalogued as *A. macrorrhizus*, has been reported as dodecaploid ($2n = 12x = 120$). The results of the ploidy analysis carried out in this work confirm that ploidy level (Fig. 3).

Another aspect showing important differences between several populations of *A. maritimus* and *A. macrorrhizus* is the phytochemical composition, specifically in the flavonoid content of these two species. The flavonoid profiles of the three populations of *A. maritimus* were very similar and indicated the presence of six identical flavonoid glycosides (Fig. 5), although there were variations on the relative percentage of each flavonoid for the different populations of *A. maritimus* (Table 3). This profile is very similar to the profiles previously reported for some asparagus genotypes of the Spanish landrace'Morado de Huétor' (Fuentes-Alventosa et al. 2007). This landrace has been described as a hybrid between *A. officinalis* and *A. maritimus* (Moreno et al. 2008). These results confirm the reliability and the stability of the phytochemical composition for a determined species. The flavonoid profile of *A. macrorrhizus* was very different (Fig. 5). The Nicotiflorin (kaempferol-rutinoside) was the principal compound identified and accounted for more than 90 % of the flavonoid content. Narcisine (Isorhamnetin-rutinoside) and rutin completed the composition of flavonoids. The low level of rutin (5 %) is characteristic of *A. macrorrhizus* (Table 3). This flavonoid account for more than 70 % of the flavonoids in the different commercial genotypes of *A. officinalis* L. (Fuentes-Alventosa et al. 2008) and more than 20 % in all populations of *A. maritimus* analyzed.

The geographical distribution of this new species is limited to the surrounding areas of the "Mar Menor" lagoon in the region of Murcia (Spain). Therefore, it may be considered an endemic species of this area. The population of *A. macrorrhizus*, formerly catalogued by mistake as *A. maritimus*, is protected in the region of Murcia within the category of "Special Interest" (Decree Law 50/2003) and was included in the category "Critically Endangered" in the Red Book of Spanish Vascular Flora 2008 (Sánchez-Gómez et al. 2008). In spite of this protection, the number of plants of this endemic species has declined in the last years. The decrease is especially significant in the area with more concentration of this species, "La Veneziola" in the north of "La Manga del Mar Menor". The urbanization of this area has been slow until recent years but the lack of available land in the rest of the "Manga del Mar Menor" has triggered this process. This suggests that the distribution area of this species was probably wider in the past. Eleven species within the genus *Asparagus* are included in the IUCN Red List of Threatened Species (IUCN 2015). The species most threatened are *A. fallax* Svent. and *A. nesiotes* Svent., which are classified as endangered species. So, without considering the extinguished *A. gharoensis* Blatter (Alam and Ali 2010), *A. macrorrhizus* is the species of the genus *Asparagus* with the highest extinction risk in the world.

Due to the high potential value as genetic resource and the extreme extinction risk of *A. macrorrhizus*, we have included this specie in the in vitro and in vivo germplasm banks located at the University of Córdoba (UCO) and the Institute for Mediterranean and Subtropical Horticulture "La Mayora" (IHSM). The plants used for the establishment of the in vivo germplasm bank were facilitated by the Official Nursery of the Directorate General for the Environment belonging to the Council of Agriculture and Water of the Region of Murcia (Spain). The micropropagation protocols developed in our previous works for other asparagus species (Carmona-Martin et al. 2014; Regalado et al. 2015) were used in the establishment of the in vitro germplasm bank. Finally, these plants have been incorporated in the UCO breeding programs.

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Compliance with ethical standards

Conflict of interest There is not any 'Conflict of Interest' in this paper.

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