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Hidden diversity in wild *Beta* taxa from Portugal: Insights from genome size and ploidy level estimations using flow cytometry

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ABSTRACT

Crop wild relatives constitute a broad pool of potentially useful genetic resources for plant breeders. The genus *Beta* L. (Amaranthaceae) is an important source of crops, primarily for sugar production. Until recently, species within Section *Beta* were mostly cytogenetically uniform, with diploidy being prevalent. Still, with the discovery of tetraploid individuals of the wild *B. macrocarpa* in the Canary Islands, a large-scale study was necessary to evaluate the cytogenetic diversity within the wild *Beta*. For that, genome size and ploidy level of *B. vulgaris* subsp. *maritima* and *B. macrocarpa* from 21 populations across Portugal mainland and islands, including all know populations of the later *taxon*, were estimated using propidium iodide flow cytometry. This work revealed a cytogenetically diverse scenario. The analyzed populations were mostly diploid, except for one population of *B. vulgaris* subsp. *maritima* that presented both diploid and tetraploid individuals, and for two populations of *B. macrocarpa* where two or three cytotypes (diploids, tetraploids and/or hexaploids) were found. The nuclear DNA content of diploid individuals was estimated as 1.44 ± 0.035 and 1.41 ± 0.027 pg/2C for *B. vulgaris* subsp. *maritima* and *B. macrocarpa*, respectively. Also, leaves of both species presented variable levels of endopolyploidy. The obtained results are discussed within the context of interspecific hybridization and cryptic diversity and constitute significant data for the conservation of these wild *Beta* crop relatives.

1. Introduction

The Euro-Mediterranean region is an important socio-economic resource of agro-diversity, harboring high variety of crops and their wild relatives (e.g., wheat, barley, olive, sugar beet; [1,2]). Crop wild relatives constitute a broad pool of potentially useful genetic resources for plant breeders. Indeed, the value of crop wild relatives has long been acknowledged and this wild resource has been used to improve crop performance since the 1940s [3–5], initially through hybridization programs, and later also through molecular technologies, significantly contributing to modern agriculture. In our days, with climate change, and eventual maladaptation of crops due to genetic impoverishment, the importance of crop wild relatives reaches a new level. Still, regardless of its recognized value, populations of crop wild relatives are under threat in the Euro-Mediterranean region and, only recently, efforts are being developed to conserve these vital resources [1,6,7].

The genus *Beta* L. (Amaranthaceae) is an important source of crops, primarily for sugar production, but also for root and leaf vegetables that have been used since antiquity [8]. *Beta vulgaris* L. subsp. *maritima* (L.) Arcang. (or wild sea beet) is considered the ancestor of all cultivated beets (e.g., leaf beet, garden beet, fodder beet and sugar beet) and is predominantly found in coastal areas around and adjacent to the Mediterranean Sea [9]. According to Hohmann et al. [10] the genus *Beta* is divided into two sections: *Beta* Transhel and *Corollinae* Ulbrich (the later including the previous section *Nanae*). Within the section *Beta*, where the crop species are included, five taxa have been recognized by different authors, namely: *B. macrocarpa* Guss., *B. patula* Ait., *B. vulgaris* subsp. *vulgaris* (all cultivated forms), *B. vulgaris* subsp. *adanensis* (Pamukc.) Ford-Lloyd & J.T. Williams and *B. vulgaris* subsp. *maritima* [11]. In Portugal, only the members of the section *Beta* can be found, namely: *B. vulgaris* subsp. *vulgaris* (cultivated and naturalized in Portugal mainland and cultivated and most probably naturalized in Azores archipelago), *B. vulgaris* subsp. *maritima* (Portugal mainland, Azores and Madeira Islands), *B. macrocarpa* (in coastal regions of center and southeast Portugal), and *B. patula* (endemic species of the Madeira archipelago) [6].

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Sugar beet, the most important crop within this genus, was developed in a very short period of time towards a high productive crop [6] and, in order to maintain the adaptability of this crop, the continuous and systematic incorporation of genetic variability from close relatives into its gene pool has been proposed [12]. Indeed, breeding programs devoted to sugar beet have introduced into its genome many traits of interest identified in other *Beta* species, especially those related with disease resistance [13–17]. Consequently, conservation programs directed to the protection of wild relatives of sugar beet have identified and prioritized several *taxa* and areas to protect [6]. These include *B. vulgaris* subsp. *maritima*, for which recent efforts enabled the successful discovery of novel genes and alleles of interest [18–20], and *B. macrocarpa* from the Canary Islands, which revealed to be cytogenetically diverse in this region, with diploid and tetraploid individuals having been identified [6,21–23].

Polyploidy, i.e., the state of having more than two complete sets of chromosomes, is a widespread phenomenon that has played a key role in the evolution and diversification of the plant kingdom [24,25]. Polyploids arise most frequently by the fusion of unreduced gametes, and may result either from the doubling of a single genome (autopolyploidy) or by the combination of two or more distinct, yet related, genomes (allopolyploidy) [26]. In breeding programs, the importance of polyploids has long been recognized, being regularly used to overcome hybridizations barriers, obtain sterile cultivars, restore fertility in hybrids, enhance pest resistance and stress tolerance and/or enhance crop vigor [15,27,28]. In sugarbeet, polyploid breeding is used mostly to increase the yield of this crop [29,30]. Indeed, tetraploids have been a staple tool for sugar beet hybrid seed production [14–17]. All *Beta* species are based on $x = 9$ chromosomes and species within Section *Beta* are presumed to be diploid ($2x = 2n = 18$ chromosomes) [6,22,31,32], except the Canary Island tetraploid forms of *B. macrocarpa* [6,21]; still cytogenetic evidence for most species is incomplete. Natural polyploids with multiple origins bear not only novel genetic diversity, but also potential increased levels of heterozygosity that can be used in breeding programs for assessing novel traits with economic interest [33–36]. With the advancement of flow cytometric applications directed to the study of plant genomes, it became possible to rapidly and easily screen the ploidy level and genome size of a high number of individuals, enabling the detection of cryptic diversity [37].

Within a broader research project aiming to delimit taxa, select appropriate wild accessions, and identify priority locations in which to establish genetic reserves of the wild *Beta* species occurring in Portugal, the objective of this study was to assess the cytogenetic diversity of wild *Beta* populations. For this, a large scale sampling of natural populations of *B. vulgaris* subsp. *maritima* and *B. macrocarpa* was performed across this region (including also the archipelagos of the Azores and Madeira), genome size was estimated using flow cytometry and ploidy levels extrapolated based on bibliographic data. The obtained results provide novel insights on the cytogenetic diversity of *Beta* wild relatives, important for breeding and conservation programs.

2. Materials and methods

2.1. Plant species

Beta L. (Amaranthaceae) is a polymorphic genus of perennial, biannual or annual species. In Portugal the most common wild taxa are *B. vulgaris* subsp. *maritima* and *B. macrocarpa*. The former taxa occurring in the coast but occasionally also in the inland, while the latter is currently confined to coastal regions of southeast Portugal, frequently growing in sympatry with *B. vulgaris* subsp. *maritima*. The populations of both species occur at undisturbed wild habitats and ruderal sites, often coastal habitats, saltworks and salt marshes. *Beta macrocarpa* is a self-compatible plant, reproducing mostly by autogamy and having low levels of observed heterozygosity [38,39]; flowering is from February to May [40]. *Beta vulgaris* subsp. *maritima* reproduces mostly by outcrossing, having high levels of observed heterozygosity [38,41]; flowering from March to September (occasionally also October) [40].

2.2. Field sampling

Twenty-one populations of *Beta vulgaris* subsp. *maritima* and five populations of *B. macrocarpa* were sampled across mainland Portugal and in Azores and Madeira archipelagos (Table S1). For *B. macrocarpa*, this sampling included all the populations and most plants from each population known in Portuguese territory. In each population, leaves from up to 26 individuals were sampled, summing a total of 114 individuals. Leaves were stored in hermetic plastic bags and maintained at 4 °C until flow cytometric analysis (performed in up to three days after sample collection). Herbarium vouchers from all the individuals sampled were collected, dehydrated and kept at the herbarium of the Tropical Botanical Garden of the Tropical Research Institute (LISC; Table S1). All specimens were identified following Gutiérrez Bustillo [42].

2.3. Genome size and DNA ploidy level estimations

Genome size and DNA ploidy levels were assessed using flow cytometry following Galbraith et al. [43] procedure for nuclear isolation. In brief, nuclear suspensions were obtained by chopping 0.5 cm^2 of fresh leaf tissue of *Beta* and 0.5 cm^2 of fresh leaf tissue of *Solanum lycopersicon* 'Stupické' or *Zea mays* 'CE.744' (internal reference standards with 2C = 1.96 pg [44] and 2C = 5.43 pg [45], respectively) with a razor blade in a Petri dish containing 1 ml of WPB buffer (0.2 M Tris–HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 °C; [46]). *Zea mays* was used only in two samples where G_0/G_1 peaks of the sample and *S. lycopersicon* overlapped. The nuclear suspension was then filtered using a 50 μm nylon mesh and 50 μg ml^{-1} of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 μg ml^{-1} of RNAse (Fluka, Buchs, Switzerland) were added to sample tubes to stain the DNA and avoid staining of double stranded RNA, respectively. Samples were analyzed in a Partec CyFlow Space flow cytometer (Partec GmbH., Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. Results were acquired using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) in the form of four graphics: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. To analyze only intact nuclei, the FL histogram was gated using a polygonal region defined in the FL vs. SS cytogram. At least 5000 particles were analyzed per sample. The mean CV values of 2C and 4C peaks of *Beta* taxa were 4.6% and 3.6%, respectively.

The holoploid genome size in mass units (2C in pg; *sensu* Greilhuber et al. [47]) was assessed using the formula: *Beta* spp. 2C nuclear DNA content (pg) = (*Beta* G_0/G_1 peak mean/reference standard G_0/G_1 peak mean) × genome size of the reference standard. DNA ploidy levels were assumed using the information available in the literature and the comparison among the results obtained. The monoploid genome size (1Cx in pg; *sensu* Greilhuber et al. [47]) of all species was also calculated by dividing the holoploid genome size by the DNA ploidy level of each taxa.

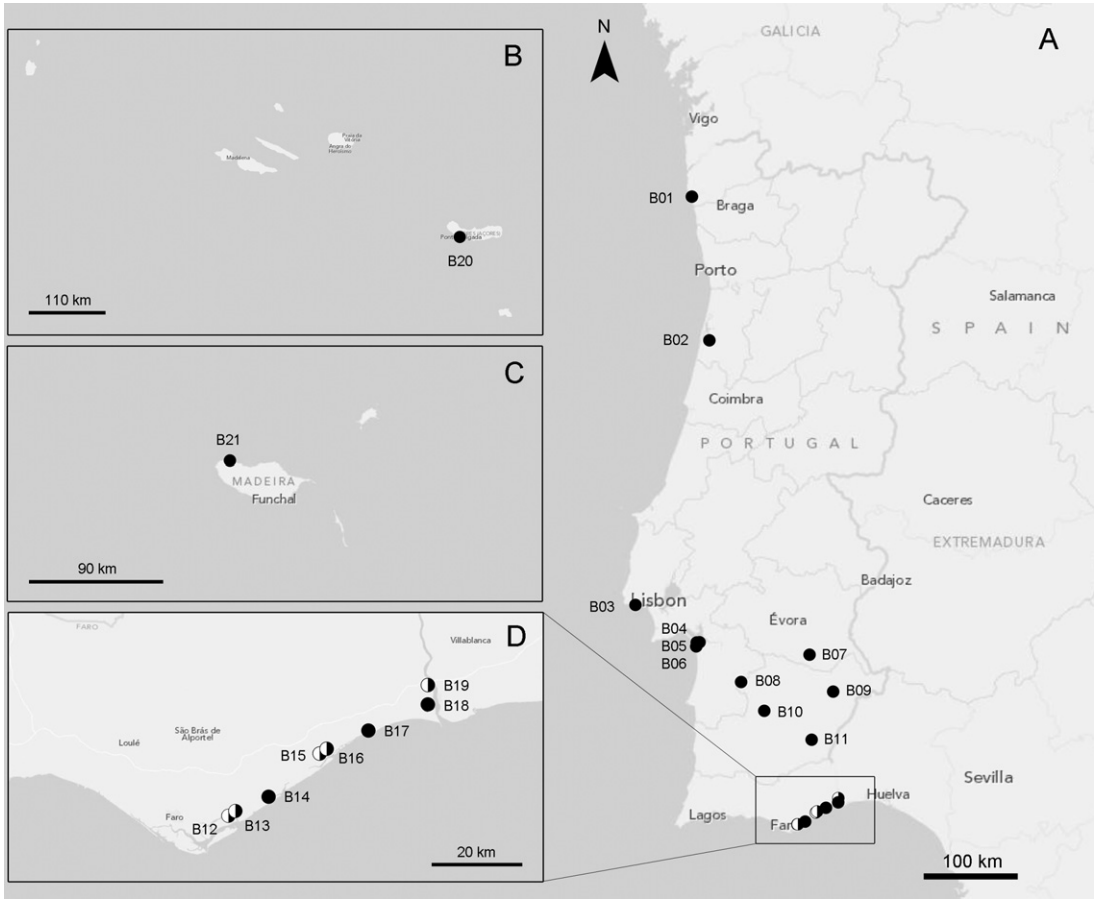

Fig. 1. Populations of *Beta macrocarpa* (white) and *B. vulgaris* subsp. *maritima* (black) sampled in Portugal mainland (A) and in Azores (B) and Madeira archipelagos (C). The region of Algarve is enlarged to show the sampling in a clearer view (D). Population localities correspond to the codes provided in Table 1.

2.4. Statistical analyses

Descriptive statistics were calculated for genome size data (mean, standard deviation of the mean, coefficient of variation and minimum and maximum values). Differences in holoploid and monoploid genome sizes among species and cytotypes were assessed with a one-way ANOVA, followed by Tukey test for multiple comparisons (SigmaPlot 12.0, Systat Software, Chicago, USA). Differences among populations of diploid *B. vulgaris* subsp. *maritima* and among population of *B. macrocarpa* were also evaluated using the same statistical approach.

3. Results and discussion

The genome size of a total of 114 individuals from 21 populations of *Beta vulgaris* subsp. *maritima* and of 33 individuals from 5 populations of *B. macrocarpa* (always growing in sympatry with *B. vulgaris* subsp. *maritima*; Table S1) was estimated using flow cytometry. These represent the first estimates for both taxa using this technique. Further individuals were analyzed for DNA ploidy level, only (Fig. 1; Table 1).

Most populations presented only one cytotype, assumed to be the most common ploidy level found in the literature, diploidy (Table 1; Fig. 2). However, in both taxa, mixed ploidy populations were detected. In *B. macrocarpa*, two populations (B13 and B15) comprised two or three cytotypes (diploid, tetraploid and/or hexaploid individuals) with diploids being more frequent than the other cytotypes (Table 1; Fig. 2); and in *B. vulgaris* subsp. *maritima*, the Azores population (B20) harbored both diploid and tetraploid individuals (Table 1; Fig. 2). The DNA ploidy level of diploid and polyploid individuals corresponded well with the genome size estimations, representing a 1:2:3 ratio for diploids, tetraploids and hexaploids, and thus full copies of the each chromosome set.

So far, with the exception of *B. macrocarpa* from Canary Islands [21,23], *Beta* was considered to be a cytogenetically homogenous genus in most of its distribution range [9,32]. With the present study, the occurrence of several populations bearing two or more cytotypes in both wild *Beta* taxa, including a new ploidy level in *B. macrocarpa* (hexaploidy), revealed that this genus is more cytogenetically diverse than previously envisaged. These populations are of major importance for conservation and crop management programs. Previous works in *B. macrocarpa* already revealed, for a confined region, the occurrence of diploid and tetraploid individuals in mixed and pure populations, with tetraploids being more common [21,23].

The studies on the origin and diversity of *B. macrocarpa* in the Canary Islands based on molecular markers suggested that the tetraploids are allopolyploids that have resulted from hybridization between *B. vulgaris* subsp. *maritima* (as maternal parent) and diploid *B. macrocarpa* (as pollen donor) [23,31]. The allopolyploid origin hypothesis is further supported by the occurrence of sympatric populations (results herein and Villain et al. [31]), by the overlap in the flowering season (in early May is the end of flowering period of *B. macrocarpa* and the start of flowering of *B. vulgaris* subsp. *maritima*, observed for example in populations B13 and B15), and by the production of viable hybrids with variable pollen fertility (41.9–83.4%) and seed viability (49.0–52.9%) after controlled pollinations [49]. Finally, hybridization and introgression of *B. vulgaris* alleles into *B. macrocarpa* have been observed in Californian populations [38]. Villain [23] suggested to rank the 4*x B. macrocarpa* from

Canary Islands as a separate taxon based on the morphological differences between the two cytotypes, and on the genetic isolation between the allopolyploid and its ancestral. In the analyzed individuals no major morphological differences were detected; still, a thorough study based on morphometric analyses is needed in the future to clarify the relationship and taxonomic status of this cytotype.

The most recent studies also support the hypothesis that the 4*x B. macrocarpa* populations from the Canary Islands result from at least two independent colonization/hybridization events in that region [23,31]. It has been shown that the western tetraploid populations have close genetic affinities with Atlantic populations of *B. vulgaris* subsp. *maritima*, whereas eastern Canarian populations are closer to Moroccan *B. vulgaris* subsp. *maritima* [44,45]. Considering the observed cytotype variation and the nature of the populations studied in this work, one of the hybridization events followed by polyploidization could have occurred in south Portugal before colonization of the western Canary Islands. Concerning the hexaploids, they can have originated from a hybridization event between a tetraploid *B. macrocarpa* and a diploid parent, either *B. macrocarpa* or *B. vulgaris* subsp. *maritima*, producing a triploid hybrid that subsequently suffered a whole genome duplication event. In other sections of *Beta*, particularly in *Corollinae*, hybrids are fixed by apomixis with only a few taxa being considered sexual species [50]. Still further studies based on molecular markers are needed to evaluate the origin of the cytotypes discovered in this study. As stated above, one locality harboring both diploid and tetraploid wild *B. vulgaris* subsp. *maritima* was detected. The presence of different ploidy levels in this taxa have been explained through hybridization events with cultivated *B. vulgaris* subsp. *vulgaris* as pollen donors, or with tetraploid *B. macrocarpa* [50–53]. Indeed, sugar beet fields occur in close vicinity (M. Romeiras, field observations). Sugar beet production is purely vegetative as the selected varieties are biennial and do not usually flower within the production period [51]. However, a few plants with low vernalization demands or resulting from crops contaminated by annual beets will flower in their first summer [12,54], increasing the likelihood of pollen flow between sugar beet and wild relatives [12,51]. Considering that commercialized sugar beet is usually diploid or triploid [55], the tetraploid *B. vulgaris* subsp. *maritima* might have originated by allopolyploidization via: (1) hybridization between a diploid sugar beet and the diploid *B. vulgaris* subsp. *maritima* and subsequent polyploidization, or (2) after the fusion of an unreduced gamete of triploid sugar beet and a reduced gamete of the diploid *B. vulgaris* subsp. *maritima*. Also, considering previous studies and despite less probable it should not be completely discarded the possibility of hybridization between diploid *B. vulgaris* subsp. *maritima* and tetraploid *B. vulgaris* subsp. *vulgaris*. In each case, this cross results in a formation of a triploid hybrid, whose unreduced pollen had to subsequently fuse with reduced gametes of the diploid *B. vulgaris* subsp. *maritima* or vice versa.

The mean nuclear DNA content of diploid individuals of *B. vulgaris* subsp. *maritima* ranged from 1.39 ± 0.039 pg/2C (population B04) to 1.49 ± 0.026 pg/2C (population B02), whereas in *B. macrocarpa*, the mean genome size of diploid individuals ranged from 1.39 ± 0.014 pg/2C (population B13) to 1.42 ± 0.035 pg/2C (population B15), being less variable than in *B. vulgaris* subsp. *maritima*. These values are in agreement with the genome size values obtained for diploid sugar beet (1.44–1.84 pg DNA/2C; [48,56–58]).

Significant differences in genome size were observed among taxa and cytotypes (Table 2). Despite representing a small difference, diploid individuals of *B. vulgaris* subsp. *maritima* presented a higher mean genome size than *B. macrocarpa* ($P < 0.05$). This

Table 1
Genome size and DNA ploidy level estimations in the populations of *Beta macrocarpa* and *B. vulgaris* subsp. *maritima* sampled in Portugal mainland and in Azores and Madeira archipelagos[a]

Pop	Species	DNA ploidy level	Genome size (2C, pg)					*n*
			Mean	SD	CV (%)	Min	Max	
B01	*Beta vulgaris* subsp. *maritima*	2*x*	1.45	0.009	0.62	1.45	1.47	6
B02	*Beta vulgaris* subsp. *maritima*	2*x*	1.49	0.026	1.74	1.46	1.52	5
B03	*Beta vulgaris* subsp. *maritima*	2*x*	1.44	0.031	2.15	1.41	1.49	17
B04	*Beta vulgaris* subsp. *maritima*	2*x*	1.39	0.029	2.09	1.35	1.41	4
B05	*Beta vulgaris* subsp. *maritima*	2*x*	1.43	0.034	2.38	1.41	1.48	4
B06	*Beta vulgaris* subsp. *maritima*	2*x*	1.44	0.029	2.01	1.42	1.46	2
B07	*Beta vulgaris* subsp. *maritima*	2*x*	1.48	–	–	–	–	1
B08	*Beta vulgaris* subsp. *maritima*	2*x*	1.47	0.030	2.04	1.43	1.50	4
B09	*Beta vulgaris* subsp. *maritima*	2*x*	1.47	0.010	0.68	1.46	1.48	4
B10	*Beta vulgaris* subsp. *maritima*	2*x*	1.46	–	–	–	–	1
B11	*Beta vulgaris* subsp. *maritima*	2*x*	1.46	0.013	0.89	1.45	1.48	3
B12	*Beta macrocarpa*	2*x*	1.41	0.018	1.28	1.38	1.43	6
	Beta vulgaris subsp. *maritima*	2*x*	1.44	0.025	1.74	1.42	1.50	8
B13	*Beta macrocarpa*	2*x*	1.39	0.014	1.01	1.37	1.40	15
		4*x*	2.82	0.029	1.03	2.79	2.84	3
		6*x*	4.23	0.049	1.16	4.20	4.26	2
	Beta vulgaris subsp. *maritima*	2*x*	1.41	0.029	2.06	1.37	1.45	10
B14	*Beta vulgaris* subsp. *maritima*	2*x*	1.44	0.036	2.50	1.39	1.49	5
B15	*Beta macrocarpa*	2*x*	1.42	0.035	2.46	1.35	1.47	11
		4*x*	2.74	–	–	–	–	1
	Beta vulgaris subsp. *maritima*	2*x*	1.45	0.037	2.55	1.35	1.50	26
B16	*Beta macrocarpa*	2*x*	1.42	0.000	0.00	1.42	1.42	2
	Beta vulgaris subsp. *maritima*	2*x*	1.42	0.029	2.04	1.39	1.47	6
B17	*Beta vulgaris* subsp. *maritima*	2*x*	1.44	0.005	0.35	1.43	1.44	2
B18	*Beta vulgaris* subsp. *maritima*	2*x*	1.43	0.007	0.49	1.42	1.43	3
B19	*Beta macrocarpa*	2*x*	1.41	0.006	0.43	1.40	1.41	3
	Beta vulgaris subsp. *maritima*	2*x*	1.41	0.010	0.71	1.40	1.42	3
B20	*Beta vulgaris* subsp. *maritima*	2*x*	1.46	0.057	3.90	1.39	1.54	7
		4*x*	2.90	0.053	1.83	2.85	2.96	3
B21	*Beta vulgaris* subsp. *maritima*	2*x*	1.41	0.008	0.57	1.40	1.42	5

[a] The following data is given for each population, taxa and DNA ploidy level: mean, standard deviation of the mean (SD), coefficient of variation (CV, %), minimum (Min) and maximum values (Max) of the holoploid genome size (2C, pg) and the total number of individuals analysed (*n*). DNA ploidy levels: 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid.

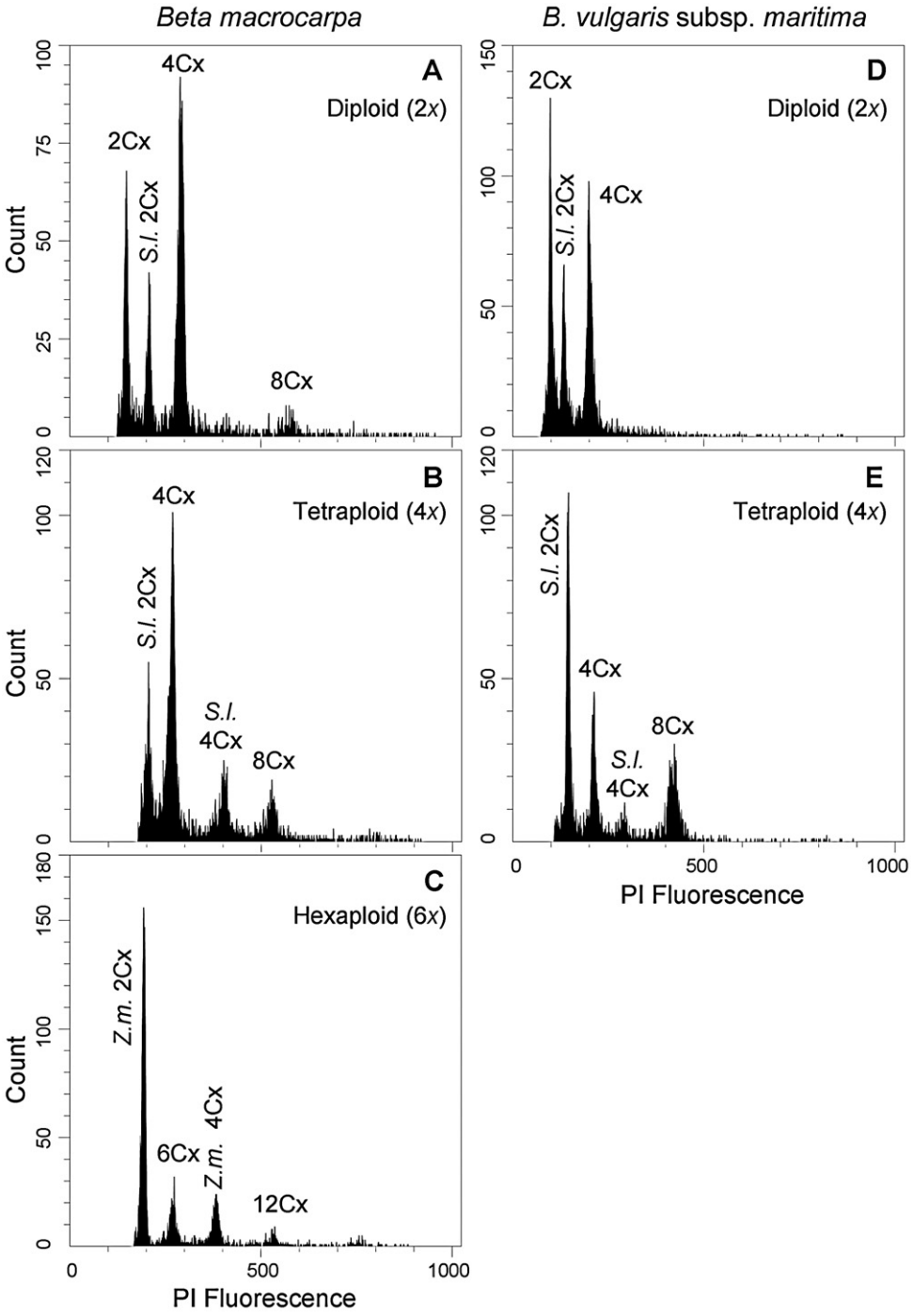

Fig. 2. Relative fluorescence histograms of propidium iodide-stained nuclei isolated from fresh leaf tissues of the internal reference standard (*S.l.*, *Solanum lycopersicon* 'Stupické', 2C = 1.96 pg DNA; or *Z.m.*, *Zea mays* 'CE.744', 2C = 5.43 pg DNA) and of the wild *Beta* species: *Beta macrocarpa* (A, diploid; B, tetraploid; and C, hexaploid individual) and *B. vulgaris* subsp. *maritima* (D, diploid; and E, tetraploid individual). In all histograms, variable levels of endopolyploidy (4Cx, 6Cx, 8Cx and/or 12Cx) can be observed.

difference was also consistent for the 1Cx value (Table 2). Despite not statistically significant, most probably because of the low sample size, the 1Cx value of the polyploid individuals of each taxa closely resembles the 1Cx value of the respective diploid taxa, indicating the absence of genome up or downsizing after polyploidization/hybridization in the taxa analyzed. Genome up or downsizing has been frequently observed in other polyploidy complexes (e.g., *Nicotiana* spp. [59]) and may result from retrotransposons activity and recombinational mechanisms, respectively [60]. The absence of this phenomenon in the wild *Beta* species may be an indication of a relatively recent emergence of the polyploid individuals and/or that the genome size was maintained by internal stabilizing mechanisms after polyploidization.

With respect to the genome size variation among diploid populations of each taxa, while for *B. macrocarpa*, no statistically significant differences were observed ($F_{4,26} = 1.273$, $P = 0.310$), for *B. vulgaris* subsp. *maritima*, the opposite occurred ($F_{18,108} = 2.694$, $P = 0.001$), with population B02 presenting significantly higher mean genome size than populations B04 and B21. A deeper analysis of the genome size variation within each population revealed that in most cases a CV value below 2.5% was observed. Still, in some populations of *B. vulgaris* subsp. *maritima*, higher CV values were

Table 2
Holoploid (2C value) and monoploid (1Cx value) genome size estimations (pg) of *Beta macrocarpa* and *B. vulgaris* subsp. *maritima* cytotypes[a]

Species	DNA ploidy level	*n*	Genome size (pg)	
			2C value	1Cx value
Beta macrocarpa	2*x*	27	1.41 ± 0.027^{a}	0.704 ± 0.013^{a}
	4*x*	4	2.80 ± 0.048^{c}	0.700 ± 0.012^{ab}
	6*x*	2	4.23 ± 0.049^{e}	0.705 ± 0.008^{ab}
Beta vulgaris subsp. *maritima*	2*x*	111	1.44 ± 0.035^{b}	0.721 ± 0.018^{b}
	4*x*	3	2.90 ± 0.053^{d}	0.724 ± 0.013^{ab}
Statistical test			$F_{4,146} = 5774.81$, $P < 0.001$	$F_{4,146} = 7.31$, $P < 0.001$

[a] The values are given as mean and standard deviation of the mean; the total number of individuals analysed (n) is also provided. DNA ploidy levels: 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid. Different letters reveal statistically significant differences at $P < 0.05$ after Tukey's test.

obtained, coinciding with sympatric populations of both taxa (ranging from 1.35 to 1.50 pg/2C in B15, for example) or the occurrence of multiple cytotypes (ranging from 1.39 to 1.54 pg/2C in B20). Such differences may support the possible occurrence of hybridization events among the two wild *Beta* species, but as they have overlapping genome sizes, further studies using molecular markers are needed to evaluate the occurrence of hybrid individuals.

Endopolyploidy was a common feature in the mature leaves of the two wild *Beta* species, with 2C, 4C, 8C (Fig. 2) and, more rarely, 16C ploidy levels being observed. No specific endopolyploid patterns with respect to taxa and/or population could be found. Previous works focused in *B. vulgaris* subsp. *vulgaris* already revealed the occurrence of this phenomenon in the genus (e.g., [48,61–63]). Still, in sugar beet, despite present in almost all organs, endopolyploid nuclei were absent from the lamina of most of the leaves. This contrasts with our results, as the mature leaves presented a marked occurrence of endopolyploid nuclei. Indeed, in the mature leaves of several species, cells with nuclear DNA content up to 16C, 32C and even 64C have been found [64–66].

The value of crop wild relatives has long been acknowledged and this wild resource has been used to improve crop performance with clear economic benefits. The results obtained in this study provided novel insights on the cytogenetic diversity of *Beta* wild relatives, with the detection of cytological diversity, namely tetraploid and hexaploid individuals that may constitute potential optimal sources for crop improvement of cultivated beets. Thus, considering the importance of wild crop relatives, it is of pivotal importance that, in the future, germplasm banks and conservation programs develop direct efforts focused in the conservation of the geographical regions encompassing the mixed-ploidy populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.02.012.

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