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Genetic Diversity and Population Structure of Wild Beets (*Beta* spp.) from the Western Iberian Peninsula and the Azores and Madeira Islands

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Abstract: In this work, using simple sequence repeat (SSR) markers, we present new insights into the genetic diversity, differentiation, and structure of *Beta vulgaris* subsp. *maritima* of western Iberia and the Azores and Madeira islands and of *B. macrocarpa* from southern Portugal. *B. macrocarpa* occurs only in southern Portugal and frequently in sympatry with *B. vulgaris* subsp. *maritima*, showing genetic introgression. *B. macrocarpa* has a better-defined structure than *B. vulgaris* subsp. *maritima*, which has a high degree of admixture. A great differentiation (F_{ST} ranging from 0.277 to 0.184) was observed among the northern populations of *B. vulgaris* subsp. *maritima*. In contrast, only a small differentiation (F_{ST} ranging from 0.000 to 0.026) was detected among the southern *B. vulgaris* subsp. *maritima* populations. The inland *B. vulgaris* subsp. *maritima* populations ("RIO" and "VMT") are distinct from each other, which also occurs with the two islands' populations ("MAD" and "AZO"). The existence of two distinct Atlantic Sea currents can explain the fact that Madeira is related to the southern populations, while the Azores is related to the northern populations. We consider that understanding the relationships existing within *Beta* spp. is key to future genetic studies and for the establishment of conservation measures. Our results show that the southern coastal areas of Portugal should be considered as a potential site for in situ conservation of the beet wild relatives. Special attention is needed in what concerns *B. macrocarpa* because this is a rare species that also occurs in a sympatric relationship with *B. vulgaris* subsp. *maritima*.

Keywords: wild beet populations; Iberian Peninsula; *F*-statistics; genetic differentiation; population structuring; admixture; sympatric populations

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1. Introduction

The genus *Beta* (*Amaranthaceae* family) is native to Europe, North Africa, and adjacent areas of the East Atlantic Coast, from about 15° N (Cabo Verde Islands) to about 58° N (south of Norway and southern Sweden) and West Asia. Climatic changes contributed to establishing the Iberian Peninsula as a differentiation center and origin of the postglacial northward expansion of plants (e.g., *Beta* genus) [1,2]. Frese et al. [3] proposed three *Beta* genepools; the primary ones include *Beta vulgaris* L. subsp. *maritima* Arcang. (sea beet),

B. vulgaris subsp. *vulgaris* (cultivated beets), and *B. macrocarpa* Guss. (large-fruited beet). Sea beet is an outcrossing species and a typical coastal taxon of the Mediterranean basin [4], but it also exists in more continental habitats [5–8]. Its distribution in mainland Portugal was updated by Monteiro et al. [9]. *B. macrocarpa* is self-compatible and occurs in the Iberian Peninsula, North Africa, Canary Islands, and West Asia (Mediterranean basin) [10]. In Portugal, it is presently only found in the south, in salt marshes and sandy soils of the Natural Park of Ria Formosa, where it is sympatric with *B. vulgaris* subsp. *maritima* [9], and in Castro Marim and Vila Real de Santo António Marsh Natural Reserve. It is at risk of genetic erosion, as its survival is linked to the traditional management of salt winning areas that can be lost with the modernization of sea salt production [11]. Therefore, it was included in the European Red List of Vascular Plants [12] and was recently classified as Vulnerable in the red list of Portuguese vascular plants [10].

Natural polyploids of *B. macrocarpa* were reported in the Canary Islands by Buttler [13] and Villain et al. [14], who also observed diploid and tetraploid forms in this species. *Beta* species can interbreed, so the tetraploid type is believed to be a natural amphidiploid between diploid *B. macrocarpa* and an unknown diploid of the *B. vulgaris* complex [15]. McFarlane [16] reported on *B. vulgaris* subsp. *Maritima* × *B. macrocarpa* hybrids, stating that they are rare due to the different flowering dates in the two species. The self-fertility of *B. macrocarpa* is an additional barrier for crossing with *B. vulgaris* subsp. *maritima*. Cytogenetic diversity within wild beet populations from Portugal was reported by Castro et al. [17]. Although most of those populations were diploid, some harbored diploids, tetraploids, and hexaploids. The regions where individuals from genetically distinct populations interbreed and form genetically mixed offsprings (hybrid zones) have been recognized to be important for evolutionary studies [18,19].

Sea beet and sugar beet have the same number of chromosomes (2n = 18) [20], but the size of their genome is different, which is 567 Mbp for sea beet [21] and 731 Mbp for sugar beet [22]. The first complete reference genome for *B. vulgaris* subsp. *vulgaris* (RefBeet) generated a broad view of genome evolution in *Beta* [22]. Funk et al. [23] published a new reference genome (EL10.1) that, together with RefBeet, provides new opportunities for studying the content and organization of the beet genome [24].

Beet crops are of great agricultural relevance. Andrello et al. [25], using SNPs, clarified the pattern of genetic structure among beet cultivated groups, and recently, Galewski and McGrath [24] identified two biological groups in the *B. vulgaris* subsp. *vulgaris* complex: the table group and a group formed by chard, fodder beet, and sugar beet (sugar and table beet are the most divergent).

The breeding gene pool of sugar beet is narrow, and it is considered that it lacks sufficient genetic variation to cope with stress. Recent results of Abou-Elwafa [26] indicate that sugar beet breeding lines they studied have a promising high degree of genetic variability concerning responses to deficit irrigation. Nonetheless, it is known that introgression subsets of wild relative diversity into crop plants are important in order to incorporate abiotic stress tolerance and other agronomic challenges valuable for increasing the resiliency of agriculture [19]. The wild beet relatives possess a high level of genetic diversity that is important to introduce useful traits in the present breeding programs. Wild beet was already used for sugar beet genetic improvement against pathogens [27], but its potential for improvement against abiotic factors has not yet been exploited. It has been proposed that the ability to accumulate compatible solutes is a breeding goal for abiotic stress tolerance [28]. Biochemical and physiological characterization of Portuguese wild beets from different habitats was performed [8,29], providing evidence of different behavior between salt marsh and inland ecotypes.

B. vulgaris subsp. *maritima* harbors genetic diversity whose patterns are determined by the mating system [30] and possibly also by marine currents when concerning coastal plant populations [1,31]. To investigate the genetic diversity found within *B. vulgaris*, molecular markers, such as microsatellites (SSRs), have been used since they yield high content of genetic data [32,33]. SSRs have also been used to investigate the pattern of

genetic structuring in natural populations [34,35] and to distinguish varieties [36], to define relationships among populations [37], and as a diagnostic tool in selective breeding [38].

Despite the studies previously performed on the *Beta* species of the Iberian Peninsula, knowledge is deficient on southwestern populations which were only partially represented. In our work, using SSRs, we present new insights into the genetic diversity, differentiation, and structure of these populations which include coastal and inland *B. vulgaris* subsp. *maritima* and *B. vulgaris* subsp. *maritima*/*B. macrocarpa* sympatric populations. In addition, the study includes *B. vulgaris* subsp. *maritima* from the Madeira and Azores islands. We envisage eliminating the existing gap and expect that our knowledge will also be useful to define locations to be considered genetic reserves. The conservation and the active management of *B. vulgaris* subsp. *maritima* and of the vulnerable *B. macrocarpa* could then be a more rational procedure.

2. Material and Methods

2.1. Plant Material and Sampling

Beta spp. were sampled from 14 different geographical locations (Figure 1): nine locations (1–3, 5, 7–11) from the Iberian Atlantic Coast, extending from the Finisterra Cape (northern Spain) to Tavira (southern Portugal); two locations (4, 6) from inland Portugal, one at a natural salt mine ("Rio Maior") the other at a pasture land, 200 km east more inland ("Vaiamonte"); two locations from Macaronesian Portuguese Archipelagos, one from the Terceira Island (Azores) (13), other from the Madeira Island (14); one location (12) from the Spain Mediterranean Coast (Gata Cape, Almeria). The Algarve locations of "Tavira" and "Quinta de Marim" (8 and 11) are both *B. macrocarpa* and *B. vulgaris* subsp. *maritima* populations living in sympatry. At these two locations, samples of *B. macrocarpa* and *B. vulgaris* subsp. *maritima* were collected. Details on the sampling locations are shown in Table S1. The field sampling was carried out during surveys performed between 2007 and 2012, following Hawkes et al. [39]. Young leaves from 23–45 plants from each Iberian population and 9 plants from Madeira and 11 plants from the Azores Islands were collected and stored at −80 °C until DNA extraction.

Figure 1. Map of sampling locations: 1—Finisterra ("FIN"); 2—Viana do Castelo ("VCA"); 3—Aveiro ("AVE"); 4—Rio Maior ("RIO"); 5—Oeiras ("OEI"); 6—Vaiamonte ("VMT"); 7—Comporta ("CMP"); 8—Tavira ("TAV"); 9—Fuseta ("FUS"); 10—Ludo ("ML"); 11—Quinta de Marim ("PM");12—Cabo da Gata, Almeria ("ALM"); 13—Terceira, Azores Island ("AZO"); 14—Porto Moniz, Madeira Island ("MAD"). *B. vulgaris* subsp. *maritima* is represented by green color; *B. macrocarpa* is represented by red color.

2.2. DNA Extraction, PCR Amplification, and Fragments Sizing

DNA was isolated from young leaves using the DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. DNA quality and concentration were visually checked on 0.8% agarose gel. DNA concentration was also estimated using a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

For *Beta* genotyping, we used the six SSR loci referred to in our previous work [8], which were developed by Richards et al. [40] from a genomic library of sugar beet, and by McGrath et al. [41] from a mapping population of an intraspecific cross between diploid sugar beet and table beet. The sequences of the primers used are provided in Table S2. PCR was conducted in a final volume of 10 µL containing 20 ng of DNA, 1× reaction buffer, 2.3 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM forward primer fluorescently labeled with WellRED dyes (D3 or D4) at the 5′-end and unlabeled reverse primers, and 0.2 units of Taq DNA polymerase (Merck). The PCR was programmed as follows: 3 min at 94 °C for the initial denaturation, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at optimum Ta for 30 s, and extension at 72 °C for 1 min. A final extension step was at 72 °C for 7 min, and the reaction was finished with a continuous cycle at 4 °C. The reactions were conducted in a Biometra TGradient thermocycler. The PCR reactions were carried out separately for each microsatellite, and mixtures of PCR products of different markers with different dyes (or distinct allele size ranges) were prepared for simultaneous detection of the amplified alleles. Subsequently, 1.0 µL of the PCR mixture was added to 24 µL formamide, and 0.5 µL fragment size standard labeled with WellRED dye D1 (DNA size standard kit, 400, Beckman Coulter, Brea, CA, USA). Capillary electrophoresis was performed to separate the PCR products using the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA). The sizes of the amplified products were determined based on an internal standard included with each sample. Data analysis was performed using the CEQ 8000 Fragment Analysis software, version 9.0, according to the manufacture's recommendations (Beckman Coulter Inc., Brea, CA, USA). Sizes of fragments were automatically calculated using the CEQ 8000 Genetic Analysis System.

2.3. Data Analysis

For the interpretation of the results, we performed analyses for the following datasets: the whole *Beta* spp. populations (northern, southern, and Islands); the northern populations of *B. vulgaris* subsp. *maritima*; the southern populations of *B. vulgaris* subsp. *maritima*; the southern populations from the Algarve region where *B. macrocarpa* ("TAVX" and "PMX") occurs in sympatry with *B. vulgaris* subsp. *maritima* ("TAV", "ML", "PM"). The latitude of the Geodesic Center of Portugal (Vila de Rei, latitude: 39°41′37″ N) was used to separate the northern populations from the southern ones. The climatic conditions are quite distinct from north to south, being rainier and colder in the north, drier and hotter in the south. Concerning the Islands, the thermal amplitude is small, with a mean temperature ca. 19 °C at Madeira and 17 °C at the Azores; the annual precipitation is higher at the Madeira location (ca. 1000 mm) than at the Azores location (ca. 820 mm).

Microchecker software v2.2.3 [42] was used for the detection of null alleles, stuttering, and allele dropout. GenAlEx 6.503 [43,44] was used to assess the genetic diversity measured as the number of alleles per locus (Na), the number of unique alleles (Npa), and the observed and expected heterozygosity (Ho and He). Allelic richness (Ar) among different populations was calculated following the rarefaction procedure [45]. The rarefaction method allows for comparisons between groups with different sample sizes. The genetic distance between each pair of individuals was calculated following the Nei methodology [46]. This analysis was performed based on the SSR genotypes with two alleles, excluding those genotypes for which a third allele was observed for one or more loci. The differentiation between the populations was estimated using Wright´s F_{ST} and Slatkin's R_{ST}, and the analysis of molecular variances (AMOVA) was calculated using GenAlEx 6.503, with 999 permutations for testing variance components. F_{ST} results were interpreted

following Del Carpio et al. [47], where 0 indicates no differentiation between populations, and a value of 1 indicates complete differentiation. Populations were considered to have great differentiation when F_{ST} values were between 0.15 and 0.25, moderate differentiation when F_{ST} values were between 0.05 and 0.15, and little differentiation when F_{ST} values were less than 0.05.

The neighbor-joining algorithm, as implemented in the DARwin software package version 6.0.12 [48], was based on a dissimilarity matrix, and the reliability of the tree topology was assessed via bootstrapping over 1000 replicates.

Regarding the PCoA, the distance matrix was calculated following Peakall and Smouse [44].

The level of genetic stratification among the studied germplasm was assessed using the STRUCTURE v.2.3.4 software [49]. This analysis was performed based on the SSR genotypes with two alleles, excluding those genotypes for which a third allele was observed for one or more *loci.* The analysis was performed considering both the admixture model and the correlated allele frequencies between populations, with values of K set from 1 to 17. The population information was incorporated into the analyses (LOCPRIOR model). Each run consisted of a burn-in period of 10^4 steps, followed by 10^6 MCMC (Monte Carlo Markov Chain) replicates assuming admixture model and correlated allele frequencies. K is the probable maximum population number that is assumed to represent and contribute to the genotypes of sampled individuals. To check the consistency of the results between runs with the same K, 17 replicates were run for each assumed K value. The approach suggested by Evanno et al. [50] was adopted to calculate the most likely value of K based on the second-order rate of change of the likelihood function with respect to K (DK). Once the number of genetic clusters was established, each individual was assigned to a cluster, and the overall membership of each sampled individual in the cluster was estimated.

3. Results

3.1. Overall Genetic Diversity

The genetic diversity of the wild beet populations was studied through a microsatellite analysis procedure similar to that one previously reported [8]. The six loci utilized (SB04, SB06, SB07, SB13, SB15, and BQ588629) amplified a total of 100 alleles, with an average of 16.7 alleles, ranging from 7 (SB13) to 25 (BQ588629) and with an average number of effective alleles (Ne) of 3.6. The polymorphism information content (PIC) ranged from 0.56 (SB13) to 0.90 (SB07) (Table 1), indicating that the used loci displayed a high level of variability and are useful diversity indicators, and it is evident that the locus SB07 displayed the highest values of Ne, He and PIC, while the locus SB13 displayed the lowest values (Table 1).

Table 1. SSRs loci used and their genetic diversity measures and *F*-statistics following Nei (1987) estimated over all wild beet populations for six SSRs loci: Na—number of alleles; Ne—number of effective alleles; Ho—observed heterozygosity; He—expected heterozygosity; PIC—polymorphism information content; F_{ST}—differentiation indices.

Locus	Na	Ne	Ho	He	PIC	F_{ST}
SB04	14	3.03	0.590	0.651	0.731	0.202
SB06	10	3.55	0.610	0.681	0.808	0.233
SB07	23	5.43	0.610	0.769	0.898	0.216
SB13	7	2.19	0.440	0.536	0.559	0.191
SB15	21	3.52	0.635	0.667	0.812	0.225
BQ588629	25	3.90	0.669	0.694	0.832	0.254

The overall genetic parameters of all the populations (Tables 2 and S3) express the existence of a considerable genetic diversity for the *B. vulgaris* subsp. *maritima* populations. The observed heterozygosity (Ho) (mean of 0.66) and the expected heterozygosity (He)

(mean of 0.71) were both high, although there was a difference between the northern and southern populations, generally higher in the south.

Table 2. Genetic diversity of the 16 wild beet populations, based on the polymorphisms of six SSRs loci: N—number of plants; Na—number of alleles; Ne—number of effective alleles; Npa—number of unique alleles to a single population; Ar—allelic richness; Ho—observed heterozygosity; He—expected heterozygosity; F—fixation index (inbreeding coefficient).

Population	N	Na	Ne	Npa	Ar	Ho	He	F
FIN	30	6.500	3.036	3	6.667	0.547	0.613	0.108
VCA	28	4.833	2.052	0	4.833	0.500	0.507	0.014
AVE	23	6.000	3.091	2	6.167	0.658	0.658	0.000
RIO	28	6.167	2.928	0	6.167	0.649	0.652	0.005
VMT	31	7.667	4.227	4	7.667	0.634	0.739	0.142
OEI	35	9.000	4.749	1	9.167	0.709	0.738	0.039
CMP	30	10.000	5.316	3	10.167	0.783	0.786	0.004
TAV	15	7.666	5.316	4	7.667	0.782	0.762	0.054
FUS	34	8.667	5.123	1	8.667	0.750	0.774	0.031
ML	30	8.333	5.022	2	8.333	0.650	0.789	0.176
PM	10	6.833	5.111	0	7.000	0.783	0.785	0.105
ALM	27	8.833	5.079	5	8.333	0.630	0.778	0.190
AZO	11	4.333	2.688	0	4.333	0.561	0.615	0.088
MAD	9	4.500	3.394	0	4.500	0.630	0.710	0.113
TAVX	30	2.500	0.899	1	2.500	0.044	0.074	0.287
PMX	19	2.333	1.092	0	2.333	0.088	0.084	−0.041

The number of alleles and the diversity (He) was lower in the northern than in the southern populations. Contrarily, the allelic richness (Ar) decreased with increased latitude. The inbreeding coefficient (F), for *B. vulgaris* subsp. *maritima*, exhibited values ranging from 0.000 ("AVE") to 0.190 ("ALM"). Values close to zero indicated random mating in the populations "VCA", "AVE", "RIO", "OEI", "CMP", "FUS" and "AZO", while substantial positive values in the "ALM", "ML" and "VMT" populations indicated interbreeding. The F negative value for "PMX" (*B. macrocarpa*) can be indicative of more heterozygotes than expected. When comparing genetic diversity of *B. vulgaris* subsp. *maritima* and *B. macrocarpa* ("TAVX" and "PMX"), it is evident that *B. macrocarpa* had lower values, which is typical of an inbreeding species. Polyploids were detected in "TAV" and "PM" locations (data not shown).

3.2. Genetic Relationships among Genotypes

The neighbor-joining tree generated from the genetic distance matrix corresponding to 390 samples illustrates the relationships among all the studied beets. When visualizing the population tree, no clear clustering emerged. Bootstrap values rarely reached 50%, indicating a low degree of resolution of the dendrogram. The exception is the set of 39 plants [16 from Quinta do Marim ("PMX") and 23 from Tavira ("TAVX")] that are in a separate branch from all the other plants, due to the fact that all of them are *B. macrocarpa*. All these beets have the same genotype (Table S3). When analyzing separately the northern and southern populations of *B. vulgaris* subsp. *maritima*, it is clear that no cluster groups were evident for the south in contrast to what was observed for the northern populations ("FIN", "VCA" and "AVE") (Figure S1A,B).

A PCoA analysis of the overall populations explained 63.60% of the variation in the first two axes, 50.75% in the first coordinate, and 12.85% in the second coordinate. Since

"PMX" and "TAVX" are *B. macrocarpa*, they were only tenuously separated from each other but were the most distant from all the other populations. It is visible that "VCA", "RIO", "MAD", and "AZO" were separated from each other and from the cloud of the remaining 10 *B. vulgaris* subsp. *maritima* populations. The two inland populations, "RIO" and "VMT", although having similar values at coordinate 1, had markedly different values at coordinate 2. The islands populations, "MAD" and "AZO", were relatively close to each other (Figure 2).

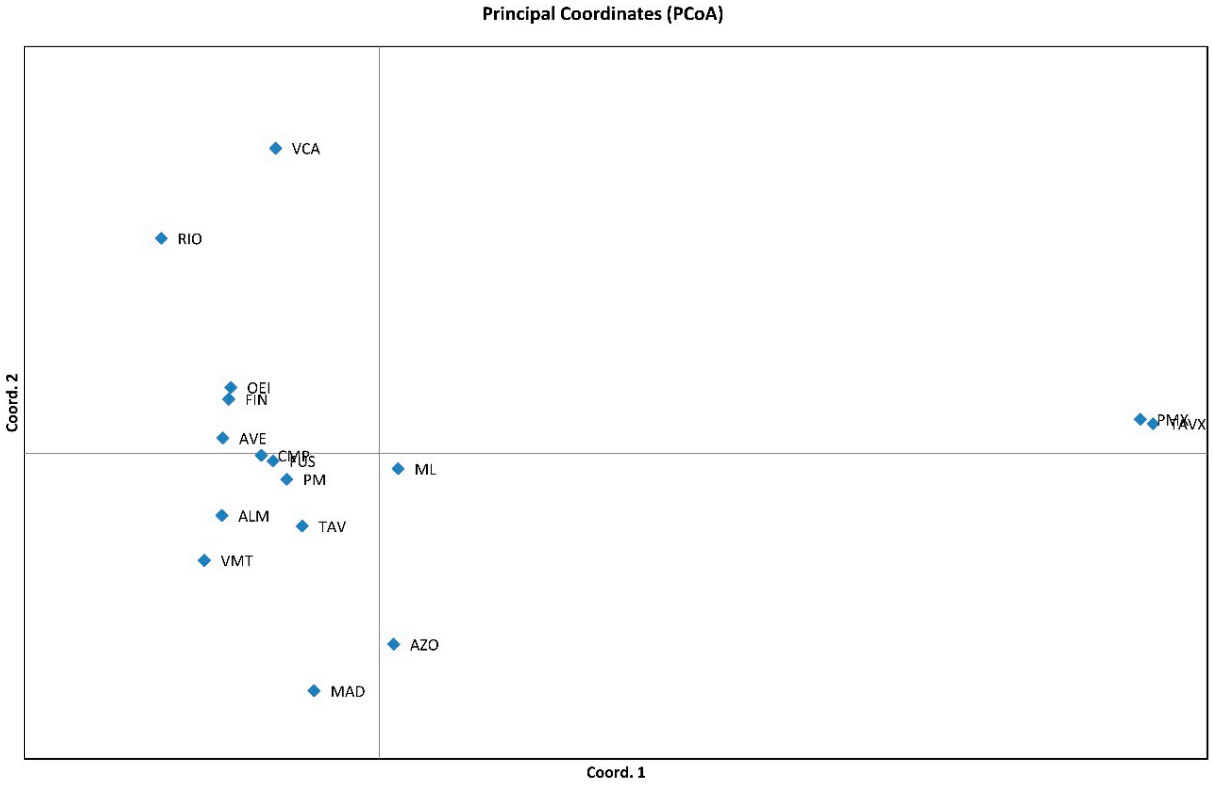

Figure 2. Scatter plot of the first and second principal coordinate based on the genetic variation of 6 SSR loci for 390 individuals of wild beet, from 16 populations. The explained variation in the first coordinate is 50.75% and in the second coordinate is 12.85%.

3.3. Differentiation of the Populations

Wright's F_{ST} and Slatkin's R_{ST} were used as a measure of the extent of the genetic differentiation among the populations. A highly pairwise differentiation (F_{ST} ranging from 0.619 to 0.346; R_{ST} ranging from 0.591 to 0.241) was detected between the *B. vulgaris* subsp. *maritima* populations and the *B. macrocarpa* populations. A great differentiation (F_{ST} ranging from 0.277 to 0.184; R_{ST} ranging from 0.294 to 0.106) was observed among the northern populations of *B. vulgaris* subsp. *maritima*. In contrast, only a small differentiation (F_{ST} ranging from 0.000 to 0.026; R_{ST} ranging from 0.005 to 0.063) was detected among the southern *B. vulgaris* subsp. *maritima* populations, suggesting the existence of close genetical affinities among these populations. The inland *B. vulgaris* subsp. *maritima* populations "RIO" and "VMT" had great differentiation values (F_{ST} = 0.156 and R_{ST} = 0.145). The same was observed (F_{ST} = 0.273 and R_{ST} = 0.178) with the two islands populations "AZO" and "MAD" (Table S4).

The analysis of molecular variance (AMOVA) indicated that the highest value of molecular variation was always found within individuals. When only the *B. vulgaris* subsp. *maritima* is considered, it is found that the value of this estimator was highest for its southern populations (88%). Considering the Algarve region, where the two *Beta* species ("TAV", "TAVX", "PM", and "PMX") live in sympatry, a variation value of 65%

was observed. AMOVA also indicated that the variation among populations of *B. vulgaris* subsp. *maritima* was 4% in the south and 23% in the north (Table 3).

Table 3. Analysis of molecular variance (AMOVA) based on 6 SSR loci, considering all wild beet populations (northern, southern, and Islands), the *B. vulgaris* subsp. *maritima* northern populations, the *B. vulgaris* subsp. *maritima* southern populations and the Algarve populations (*B. vulgaris* subsp. *maritima*/*B. macrocarpa* living in sympatry).

Source of Variation	df	Sum of Squares	Variance Components	Variation (%)
All populations				
Among populations	15	397.135	0.505	21
Among individuals	374	771.772	0.161	7
Within individuals	390	679.000	1.741	72
Northern populations				
Among populations	2	61.238	0.533	23
Among individuals	78	146.824	0.102	4
Within individuals	81	136.000	1.679	73
Southern populations				
Among populations	6	41.988	0.088	4
Among individuals	174	442.261	0.207	8
Within individuals	181	385.000	2.127	88
Algarve populations				
Among populations	5	140.259	0.588	27
Among individuals	132	234.969	0.169	8
Within individuals	138	199.000	1.442	65

With 1000 permutations; $p < 0.001$.

3.4. Genetic Structure

The Bayesian approach indicated that the most likely number of genetic clusters was K = 2 (Delta K = 131.81), while the second-best solution was K = 3 (Delta K = 2.88), and the third solution was K = 5 (Delta K = 1.45) (Figures 3 and S2). Based on the results of the STRUCTURE analysis, it was considered that accession with a score higher than 0.80 was pure, while that with a lower score was considered to be admixed.

The two groups assigned at K = 2 correspond to *B. vulgaris* subsp. *maritima* populations (red color) and *B. macrocarpa* (green color). *B. vulgaris* subsp. *maritima* and *B. macrocarpa* were living in sympatry at Tavira and Quinta de Marim.

At k = 3, the clustering of *B. vulgaris* subsp. *maritima* populations is represented by two colors, green and blue, and *B. macrocarpa* by the red color. The *B. vulgaris* subsp. *maritima* populations that we considered pure were "FIN", "VMT", "CMP", "TAV", "FUS", "PM", "ALM" and "MAD", for the blue color, "VCA" and "RIO" for the green color, and the *B. macrocarpa* populations "TAVX" and "PMX" for the red color. All other *B. vulgaris* subsp. *maritima* populations had some degree of admixture. The effect of introgression between *B. vulgaris* subsp. *maritima* and *B. macrocarpa* was also evident, with higher intensity in population "ML". An important observation is that the two inland *B. vulgaris* subsp. *maritima* populations "RIO" and "VMT" were distinct from each other, which also occurred with the islands' populations "AZO" and MAD". K = 5 further indicates that the northern *B. vulgaris* subsp. *maritima* populations were less admixed than the southern ones. The genetic introgression between *B. vulgaris* subsp. *maritima* and *B. macrocarpa* was also revealed at "ML", "TAV", and "PM".

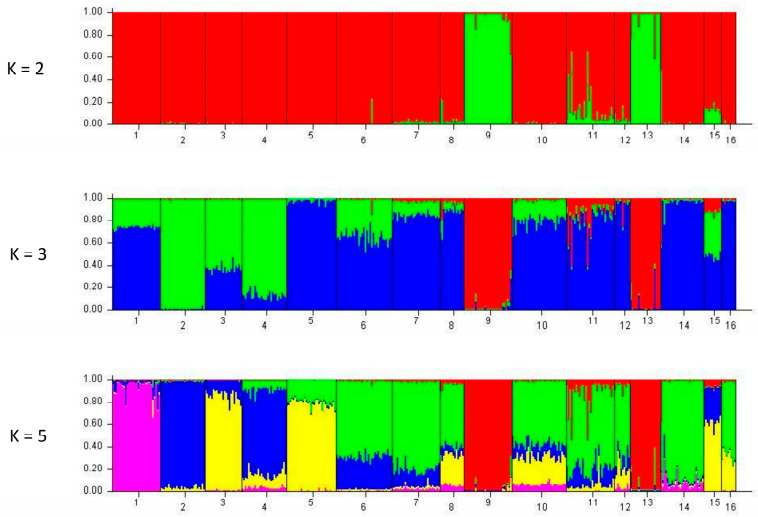

Figure 3. Graphical display of the results of the STRUCTURE analysis, inferred at K = 2, K = 3, and K = 5. Each population accession is represented by a vertical line segmented into a number of colors identical to the K number: 1—Finisterra ("FIN"); 2—Viana do Castelo ("VCA"); 3—Aveiro ("AVE"); 4—Rio Maior ("RIO"); 5—Vaiamonte ("VMT"); 6—Oeiras ("OEI"); 7—Comporta ("CMP"); 8—Tavira ("TAV", *B. vulgaris* subsp. *maritima*); 9—Tavira ("TAVX", *B. macrocarpa*); 10—Fuseta ("FUS"); 11—Ludo ("ML",); 12—Quinta de Marim ("PM", *B. vulgaris* subsp. *maritima*); 13—Quinta de Marim ("PMX", *B. macrocarpa*); 14—Almeria ("ALM"); 15—Azores ("AZO"); 16—Madeira ("MAD"). At K = 2, *B. vulgaris* subsp. *maritima* is represented by (red color) and *B. macrocarpa* by green color; at K = 3 and K = 5, *B. macrocarpa* is represented by red color.

4. Discussion

The genetic diversity of wild beet was previously studied using SSRs—namely, in some regions of the Iberian Peninsula [1]. However, studies concerning Portugal were only partial, and in our work, we present new insights into the diversity of wild beet by studying coastal, inland, and islands *B. vulgaris* subsp. *maritima* populations and also sympatric *B. vulgaris* subsp. *maritima*/*B. macrocarpa* populations from the southern coast of the country.

The observed high level of genetic diversity for *B. vulgaris* subsp. *maritima* and the low level of genetic diversity for *B. macrocarpa* could be explained by the distinct reproductive systems (outcrossing in *B. vulgaris* subsp. *maritima* and selfing pollination in *B. macrocarpa*). Outcrossing populations had greater allelic diversity, higher levels of heterozygosity, and showed lesser differentiation among populations [51]. These observations are in accordance with previous work [1,32,33,52], which also showed higher genetic diversity for the southern populations than for the northern ones, in several European regions and North Africa [1,33]. The Almeria ("ALM") value, the only Mediterranean sample that we studied, presented a similar He to that of our southern populations, which contrasts with the results obtained by Richards et al. [33] for the Mediterranean wild beet. Future studies, including more samples from the Mediterranean coast, are needed to evaluate the significance of this discrepancy.

Considering the inbreeding coefficient (F), its greater than zero value observed for "FIN", "VMT", "ALM", and "MAD" could be due to their geographic isolation. "FIN" and "ALM" were at Langosteira beach (Finisterra, Spain) and Cabo da Gata beach (Almeria, Spain), respectively, and "VMT" is an inland ruderal and isolated population, and "MAD" is an island population. The high F value for the *B. vulgaris* subsp. *maritima* "ML" is interesting since it is not an isolated population; furthermore, we did not detect in the field the presence of *B. macrocarpa*, but the results show that there was hybridization with this species (Figure 3). Possibly, this is an influence from the *B. macrocarpa* "PMX" population, which is located only ca. 5 Km away.

Several studies have revealed shared DNA polymorphisms between closed related species [17,53–56], with natural interspecific hybridization being estimated as approximately 25% of all plant species [57]. Genetic admixture and interspecific hybridization also occurred in the *B. vulgaris* subsp. *maritima* populations in the southern Portuguese region. The introgression was mostly detected in zones of sympatric distribution, meaning that no genetic barriers exist between *B. vulgaris* subsp. *maritima* and *B. macrocarpa* present in these locations. Indeed, it was shown that *Beta* section hybridization presents no major problems [58] and that genes may be exchanged uni- or bidirectionally [59]. Natural hybrids involving *B. macrocarpa* were reported by McFarlane [16], who stated that they are rare only due to differing flowering dates of parental species. One of the most widely recognized short-term benefits of admixture is heterosis and the emergence of novel phenotypes, which may increase the overall population genetic variance, resulting in a higher capacity to respond to selection pressure [60] fitness and adaptive responses [61,62].

Since *B. vulgaris* subsp. *maritima* from "TAV" and "ALM" locations had the highest number of unique alleles, these populations may need particular attention when defining a conservative strategy for the *Beta* species, as was previously proposed by Roussel et al. [62].

The *B. macrocarpa* populations were rather distinct from all the other populations when considering the genetic relationships among the genotypes, and all their 39 accessions had a close genetic relationship among them. It is evident that this resulted from the fact that these populations were the most divergent of all our studied beets. The lack of clear clustering patterns for the remaining *B. vulgaris* subsp. *maritima* populations is a consequence of the outcrossing biology of this beet, similarly to what was referred to for other species [63,64].

F_{ST} and R_{ST} are useful differentiation estimators, commonly used to describe population structuring [65,66]. A high degree of genetic differentiation was found when comparing *B. macrocarpa* with *B. vulgaris* subsp. *maritima*. *B. macrocarpa* was also shown to have a very low genetic variation, as a consequence of its reproductive system. Previous work for these two species reached identical conclusions when using nuclear SSRs and DArT markers [1,52]. The F_{ST} value we observed for the northern *B. vulgaris* subsp. *maritima* populations could be the result of a certain degree of geographic isolation of these populations. However, the same did not apply to *B. vulgaris* subsp. *maritima* southern populations. Therefore, according to the standards of Del Carpio et al. [47] and Mohammadi and Prasanna [67], the northern populations had a great differentiation that was not observed in the southern populations.

In our study, the clinal variation was only visible at K = 3 and K = 5, which contrasts with Andrello et al. [52] results that showed clinal variation at K = 2.

AMOVA results demonstrated that molecular variation was mainly found within individuals, as expected for an outcrossing species [24,63,68].

The distinct mating system of the two species (*B. vulgaris* subsp. *maritima* and *B. macrocarpa*) is also the cause of the distinct genetic structure we observed for each of the three different K values used. It is known that the breeding system and the gene flow have major evolutionary effects on population genetic structure [69,70]. It is also evident the effect of introgression between *B. vulgaris* subsp. *maritima* and *B. macrocarpa*, with higher intensity in the "ML" population, visible for all values of K.

Contrarily to the coastal beets, the origin of the Portuguese inland beets is presently unknown. It has been suggested that ruderal beets can result from cultivar seed escape [7], a hypothesis also supported by Saccomani et al. [69] for Italian ruderal beets. However, the "VMT" beets cannot be viewed as the result of seed escape since there is no report of sugar beet cultivation nearby [8]. We considered the possibility of some other anthropogenic influence. To clarify its origin as well as the origin of the "RIO" (also an inland beet), cytoplasmic DNA markers should be used in order to unravel their maternal origin, because the seed dispersal is a determinant of the genetic structure [70].

Concerning the populations from the islands, our STRUCTURE results (K = 3 and K = 5) indicate that while "MAD" was related to the southern populations, "AZO" was

related to the northern populations (particularly to the "AVE" population). Admitting sea current dispersal suggested by Leys et al. [1], it is interesting to note that they resulted from two distinct Atlantic sea currents.

5. Conclusions

SSRs are useful tools for studying genetic diversity, even the limited number we used. We could discriminate the beet populations of northern and southern western Iberia and of the Madeira and Azores islands. The importance of some of these populations justifies the concern about their conservation. When selecting conservation sites one must consider the population´s total diversity and the allelic richness [71]. The *B. vulgaris* subsp. *maritima* southern populations were inferred to have the greatest contribution to total diversity. In addition, the beets of the southern coast of Portugal (particularly, from the Algarve region) should be further investigated as potential sites for in situ conservation due to the *B. vulgaris* subsp. *maritima*/*B. macrocarpa* genetic introgression. Furthermore, since *B. macrocarpa* is a threatened species, red listed as Vulnerable in Portugal, only present at salt marshes, it needs special attention for its conservation.

Wild beets are relevant genetic reservoirs for sugar beet improvement. Therefore, from the breeding point of view, conducting a close study on a particular population would be advisable when carrying out crop genetic improvement. Drought is a major constraint for sugar beet production, and the specific habitats of some western Iberia wild beets indicate they could have important characteristics for resistance to this and other abiotic stresses. A deeper study to fully evaluate the agronomic potential of these native populations is justified.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13110593/s1, Table S1: Sampling locations, Table S2: Primer sequence information, Table S3: SSR screening of 390 *Beta* spp. accessions using 6 loci, Table S4: Pairwise F_{ST} and R_{ST} matrix for all the populations, Figure S1: Neighbor-joining dendrogram for northern (A) and southern (B) populations, Figure S2: Exploration of K values for STRUCTURE analysis of *Beta* spp. accessions.

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