Assessing the Diversity of Sea Beet (*Beta vulgaris* L. ssp. *maritima*) Populations

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ABSTRACT

Beta vulgaris L. subsp. *maritima* (L.) Arcang., sea beet, is a morphologically and genetically variable species, belonging to beet primary gene-pool. This crop wild relative is a valuable genetic resource for resistance improvement in beets and could play an important role in crop yield sustainability. Eleven Madeiran sea beet populations were characterized using morphological descriptors and genetic markers. Our goal was to evaluate these populations as a potential source of valuable genetic material. Morphological characterization showed a high quantitative variation among populations. Plant height and inflorescence height parameters had the highest influence in the separation of populations. Molecular analysis was performed with polymorphic SSRs to determine genetic variability between populations. Both PCA and PCoA revealed three clusters that separated the populations according to morphological and genetic traits, respectively. This study contributes to the knowledge of sea beet diversity in Madeira's archipelago and to the perception that the islands' specific environmental conditions influence its genetic variability, making these populations a possible gene source for sugar beet breeding programs

Keywords: Crop wild relatives, Genetic resources, Morphological traits, SSRs, Sugar beet.

INTRODUCTION

The screening of adaptive traits diversity, found in Crop Wild Relatives (CWR), is an important target of many crop breeding programs (Labokas *et al.*, 2018). A pressing need for conservation of useful genetic diversity for crop plants has led to prioritization and increased investment in the survey, sampling, and evaluation of its wild relatives (Arzani and Ashraf, 2016; Labokas *et al.*, 2018; Khoury *et al.*, 2019).

The *Beta vulgaris* subsp. *maritima* (L.) Arcang., the ancestral of all domesticated beets (Panella and Lewellen, 2007; Castro *et al.*, 2013) and commonly known as wild or sea beet, is widely distributed in Madeira Archipelago (Borges *et al.*, 2008; Vincent *et al.*, 2013). The species is a CWR of interest, that belongs to beets' primary gene pool, and a possible source of useful traits (Vincent *et al.*, 2013) that were lost as a result of the domestication process of white fodder beet (Panella and Lewellen, 2007). The genus *Beta* includes eleven CWR, of which three are present in Madeira archipelago, and one of these, *Beta patula* Aiton, is endemic. In 2013, the *Beta* genus was included in a global priority conservation list of 92 CWR genus (Vincent *et al.*, 2013). The improvement of beets for agricultural purposes, mainly target yield, and economically valuable traits determining the erosion of genetic diversity

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and disappearance of adaptive skills for environmental changes, disease or pest resistance (Panella and Lewellen, 2007; Matesanz and Milla, 2018). Sea beet populations show genetic variability (Boudry _et al._, 2002), presenting skills for adaptation in environmentally challenging habitats and resistance to diseases caused by viruses, fungi, or other plagues (Biancardi _et al._, 2012a), which are useful for breeding purposes and crop adaptation (Panella and Lewellen, 2007). Until 2011, a total of 21 useful traits were transferred from sea beet to sugar beet, using normal breeding methods (Biancardi _et al._, 2012b).

Sea beet has a large distribution, growing in the Atlantic coasts of western Europe, Scandinavia, Macaronesia, coastal areas of the Mediterranean Basin, the Middle East, and the Indian subcontinent. Inland populations can be found in the Mediterranean basin, where they prefer desertic areas and clay soils (Andrello _et al._, 2016; Bartolucci _et al._, 2018). Populations of sea beet can occupy areas where water is scarce and soil salinity high, creating a selective pressure that prompts its adaptation (Ribeiro _et al._, 2016). The biological cycle of sea beet can vary greatly, as populations can have a mixture of annual, biennial, and perennial plants and often contain several genotypes. Some of them bloom after the first/second year, others every year, after a long vegetative phase, and some after a not specified number of years. The presence of different biological cycles helps the species survive in extreme conditions, showing that the behavior of an individual or population is a response to the environment of the occurrence site (Letschert and Frese, 1993). This species reproduces by outcrossing (Castro _et al._, 2013) and it can successfully hybridize with cultivated varieties of the leaf or root beet (Bartsch and Schmidt, 1997). In Madeira Island, sea beet grows on the top of cliffs above the sea. In Porto Santo Island (Madeira archipelago), sea beet can be found as inland populations, but due to the reduced geographical area of the island, these populations suffer great influence from the sea. This study aimed to assess: (1) The heterogeneity and variability of sea beet populations of Madeira's archipelago, since this CWR has not yet been studied or explored in this region, and (2) Phenotypic and genotypic variability of different populations.

MATERIALS AND METHODS

Population Survey and Morphological Characterization

Eleven sea beet populations were identified and sampled in the year 2017. There were seven populations from Madeira Island and four from Porto Santo Island (Table 1, Figure 1). Table 1 also shows the mean values of climatic parameters of the locations where these populations occur. All accessible and suitable sites for beet CWR occurrence were surveyed in both islands. Fourteen plants of each population were randomly collected in the field during the phenological stage of "full flowering" (spring-summer), and sampling was carried out to represent the maximum phenotypic variability of the populations. Populations were considered distinct if they were more than 15 km apart or separated by evident physical barriers (Figure 1) (Stevanato _et al._, 2013).

According to ecogeographic distribution, seven populations (POPs 1, 2, 3, 5, 6, 8, and 11) occur in ruderal or abandoned places. The remaining populations occur in wild places with different levels of human pressure (POPs 4, 7, 9, and 10).

In live plants, eleven morphological quantitative traits were measured, according to similar studies and CPVO (Community Plant Variety Office) Protocol Guidelines for beet leaf (Letschert and Frese, 1993; Srivastava _et al._, 2000; CPVO Technical Protocol for leaf beet, 2015), namely, Number of Basal Stems (NBS, n°); Plant Height (PH, cm); Inflorescence Height (IH, cm): Distance of the first Branch from the Basis (DBB, cm); the Number of Branches (NB, n°); Leaf

Table 1. Identification and geographic information of the eleven sampled populations of *B. vulgaris* subsp. *maritima* and climate parameters in the sampling areas.

POP[a]	Genebank code	Place	Mean temp (°C)	Mean max temp (°C)	Mean min temp (°C)	Mean RH (%)	Total precipitation (mm)
1	ISOP 3114	Ponta do Pargo	18.6	21.6	15.6	76	394.6
2	ISOP 3113	Porto Moniz	19.8	21.6	17.9	72	490.8
3	ISOP 3115	Praia Formosa	20.9	23.5	18.1	66	219.3
4	ISOP 3105	Ponta de São Lourenço	19.3	21.2	17.4	75	326.1
5	ISOP 3106	Garajau (Cristo Rei)	20.2	22.5	17.9	68	480.1
6	ISOP 3107	Ponta Oliveira	20.2	22.5	17.9	68	480.1
7	ISOP 3108 ISOP 3109	Farol/Desembarcadouro islets	19.3	21.2	17.4	75	326.1
8	ISOP 3111	Serra de Fora	19.7	22.3	17.1	76	366.9
9	ISOP 2549	Praia Deserta	19.7	22.3	17.1	76	366.9
10	INSC 4082	Baixa dos Barbeiros	19.7	22.3	17.1	76	366.9
11	INSC 4083	Aeroporto (PXO)	19.7	22.3	17.1	76	366.9

[a] population
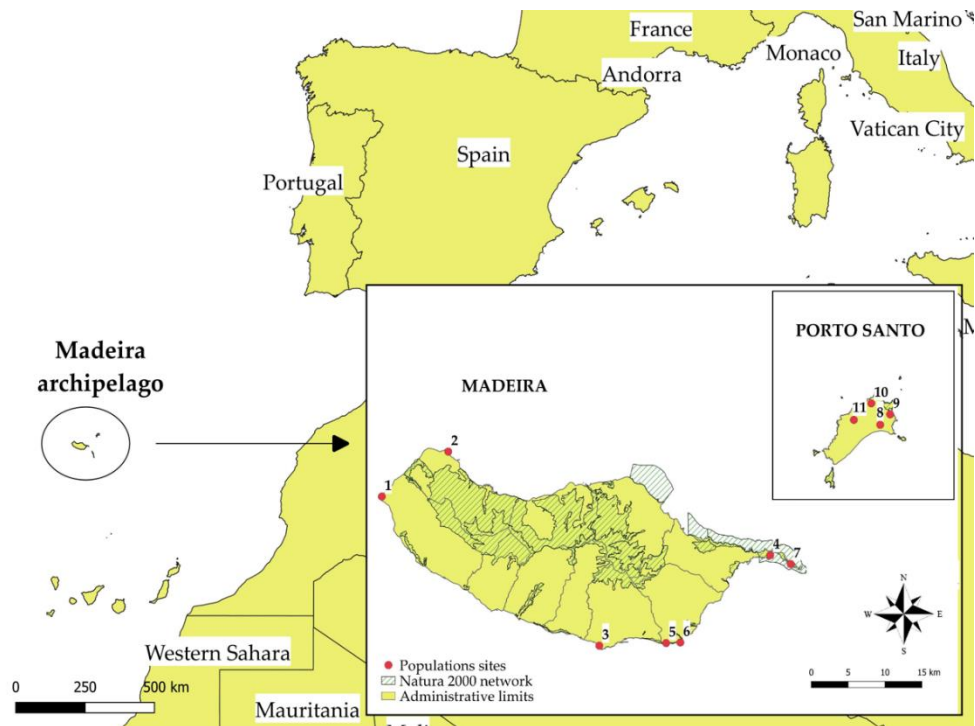
Figure 1. Map showing sites from Madeira and Porto Santo Islands where eleven populations of *Beta vulgaris* subsp. *maritima* were sampled. Numbers represent the CWR population number.

Width (LW, cm); Leaf Length (LL, cm); Petiole Width (PW, mm); Petiole Length (PL, mm); Stem Diameter (SD, mm); and average Number of Glomerulus per branch (NG, n°). Some CPVO leaves traits were not used since their protocol was developed for cultivating beets and were not detectable in sea beets. PH was recorded from plant collar to the top of the highest inflorescence. IH was recorded on the highest inflorescence branch. DBB was recorded considering only the branches with flowers. To determine morphological traits on leaves, fully developed, and healthy ones were chosen

Table 2. SSR markers designations and traits associated with genetic analysis of *B. vulgaris* subsp. *maritima*.

Type of marker	Molecular markers	Traits [a]	References
SSR	2KWS (SSR2)	Leaf Na$^+$, Root Na$^+$, WSC, ECS; SC + WSC in saline and non-saline conditions.	Abbasi *et al.* (2015)
SSR	BQ584037	Phosphatidylglycerolphosphate synthase.	McGrath *et al.* (2007)
SSR	BQ588629	BSD domain-containing protein (Pfam PF03909).	McGrath *et al.* (2007)
SSR	FDSB1027	Sugar and WSY; saline responses.	Abbasi *et al.* (2015)
EST-SSR	FDSB1250	Hydrolase family protein (Pfam PF00657); GDSL esterase/lipase.	NCBI
SSR	SB04	Anonymous SSR.	--------------
SSR	SB13	Growth-regulating factor 7.	NCBI
SSR	SB15	Sugar yield-related traits: SY, WSY, RY, WSC, ECS; saline responses.	Abbasi *et al.* (2015)

[a] EST: Expressed Sequence Tag; SC: Sugar Content; WSC: White Sugar Content; ECS: Extraction Coefficient of Sugar; WSY: White Sugar Yield; RY: Root Yield, SY: Sugar Yield.

from the base of the plant. SD was measured on the plant collar. NG was the result of a mean of the glomerulus on the 3rd biggest branch for each plant. Leaves´ traits and stem diameter were measured with digital pachymeter and the other traits with a standard measuring tape. Leaf samples were dried and preserved at room temperature in plastic bags sealed under vacuum. Seeds were collected at the same moment as the leaves collection or later when they were mature and dry (from 60 plants per population at maximum) and were included in the ISOPlexis Genebank germplasm collection. For populations 10 and 11, seed collection was not possible since no mature seeds were observed during the survey.

Genetic Analysis

Eight polymorphic SSRs (Simple Sequence Repeats) markers, developed for *B. vulgaris* subsp. *maritima* genome sequence, were selected based on associated traits. In Table 2, marker designation and associated traits are shown. Fifty-five individuals (5 individuals from each population) were selected based on their morphological variation (individuals with the biggest intermediate and smallest size measured), as an attempt to link morphological phenotypes to genetic patterns. DNA was extracted from dried leaves, as described by Shiaoman Chao and Daryl Somers' protocol (Chao *et al.*, 2012), with modifications made by the substitution of isopropanol for chloroform: isoamyl acid (24:1), and centrifuged for 10 minutes at 12,000 rpm. Recovered the supernatant, 360 μL of isopropanol was added, mixed, and left to precipitate for 15 minutes. Pellet was resuspended in 100 μL of Tris-EDTA (pH 8) and left overnight at 4 ℃. The supernatant was recovered and stored at -20℃. DNA was amplified in a 25 μL volume sample with 12.5 μL Thermo Scientific Phusion HF PCR Master Mix, 2 μL prime, 5.5 μL Milli-Q water and 5 μL of DNA. For BQ584037 marker amplification, 1 μL of DMSO was added. BIOER Life ECO thermal cycler was used for sample amplification, with the following PCR conditions: 98℃ for 1 minute, 40 cycles of 98℃ for 10 seconds, 30 seconds at annealing temperature, and 72℃ for 30 seconds; followed by 72℃ for 10 minutes. PCR products were analyzed by separation in 5% polyacrylamide gel for higher resolution results.

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Data Analysis

Morphological Analysis

Morphological characterization data were subjected to ANOVA using the software SPSS v.24 (Statistical Package for the Social Science). One-way ANOVA was applied to evaluate differences between the populations for the morphological traits. Principal Component Analysis (PCA) was performed to explore the observed variability and relatedness of the populations, using MVSP software (Multi Variate Statistical Package). For this PCA analysis, the leaf length/leaf width ratio was calculated. A discriminant analysis was performed to ascertain the robustness of the three clusters that were created to group the variability of the populations. A One-Way ANOVA analysis followed by a Tukey HSD mean comparison *post hoc* test was used to test for significant differences between population clusters identified in the PCA.

Molecular Analysis

Amplification results were analyzed using Fingerprinting II Informatix software (Bio-Rad Laboratories). Heterozygosity values [using Levene's and Nei's algorithms (Levene, 1949; Nei, 1973)], Fixation Index (F_{IS}) using Wright's formula (Wright, 1978) and Shannon-Wiener's Index (Shannon and Weaver, 1949) for each population were calculated, using POPGENE version 1.31 software. F_{IS} gives us the inbreeding coefficient, ranging from 0 to 1, where high values imply a considerable degree of inbreeding and low values indicate that populations are at or near Hardy-Weinberg equilibrium. Polymorphic Information Content (PIC) was calculated with the formula: $1-\sum (Pi)^2$ (where P is the allele frequency for the *i* allele). For a visual ordination of variation patterns, Principal Coordinates Analysis (PCoA) was performed using MVSP software, with data processed using Gower General Similarity Coefficient and transformed using log(e).

RESULTS

Morphological Characterization and Analysis

The analysis of variance revealed significant differences amongst the eleven populations for the morphological traits (Table 3). Two populations present the highest values in four morphological traits, namely, POP 2, for traits PH, IH, LL and PL, and POP 3, for NB, LW, PW, and SD traits. These are followed by NBS and DBB for POP 1 and NG for POP 5. The population that stands out with the lowest values for the morphological characters PH, IH, DBB, NB, and NG is POP 7. POP 10 also presents the lowest values for NBS, LW, LL, and PL. Three other populations have the lowest values for only one trait, namely, POP 4 for SD, POP 5 for NBS and POP 9 for PW. Populations from Madeira Island have higher values for all morphological traits, with POPs 2 and 3 standing out (Table 3). Both populations occur in similar ruderal places.

Regarding the PCA (Figure 2), three population clusters were outlined. Discriminant analysis showed that 100% of the populations were correctly classified to each cluster, and data cross-validation confirmed that 90.9% of the cases were correctly classified. The mean comparisons for morphological traits of the three clusters are summarised in Table 4.

Populations of cluster III have significantly higher values and variability for all morphological traits than populations from the remaining clusters. Cluster I grouped the populations from the eastern part of Madeira and two populations from Porto Santo. Cluster II holds the two populations of Porto Santo that are intermediate (regarding plant height). Cluster III aggregates all the populations from the western and southern parts of

Table 3. Mean comparisons of morphological traits for the eleven sea beet populations.[a]

POP	Number of basal stems	Plant height	Inflorescence height	Distance of the first branch from the basis	Number of branches	Leaf width	Leaf length	Petiole width	Petiole length	Stem diameter	Average number of glomerulus per branch
1	**8.43±2.63^{d}**	68.04±5.76^{ef}	46.79±5.23^{de}	**21.25±3.70^{c}**	18.86±2.19^{cd}	4.21±0.41^{cd}	7.71±0.71^{cd}	5.06±0.62^{cde}	30.26±4.40^{bcd}	9.01±1.17^{b}	30.98±3.92^{cd}
2	7.29±1.50^{bcd}	**79.79±6.24^{e}**	**58.88±7.73^{e}**	20.91±4.35^{c}	19.21±2.60^{d}	5.63±0.76^{d}	**10.85±0.91^{e}**	5.75±0.59^{cde}	**55.84±7.45^{e}**	7.08±1.02^{ab}	26.57±3.25^{abcd}
3	2.00±0.38^{ab}	71.71±5.88^{ef}	52.93±5.92^{e}	18.79±3.37^{bc}	**19.43±2.73^{d}**	**5.67±0.58^{d}**	10.78±1.04^{de}	**6.61±0.73^{e}**	38.86±9.20^{de}	**11.12±1.79^{b}**	29.14±2.34^{bcd}
4	7.86±1.10^{cd}	13.21±1.64^{ab}	10.25±1.59^{abc}	2.96±1.49^{a}	4.07±0.73^{ab}	1.59±0.29^{ab}	3.87±0.74^{ab}	2.36±0.36^{ab}	12.63±4.55^{abc}	2.99±0.28^{a}	17.14±1.60^{ab}
5	1.00±0.00^{a}	56.75±4.38^{de}	44.86±5.45^{de}	11.89±3.05^{abc}	12.21±2.69^{bcd}	3.89±0.44^{cd}	7.12±0.68^{c}	6.22±0.87^{de}	33.53±7.08^{cde}	6.95±0.81^{ab}	**34.69±2.93^{d}**
6	3.36±1.27^{abcd}	64.57±4.28^{ef}	47.04±4.56^{de}	17.54±2.99^{bc}	10.57±1.34^{abc}	3.38±0.32^{bc}	7.14±0.45^{c}	4.18±0.38^{abcd}	35.32±5.91^{cde}	8.91±1.51^{b}	32.26±2.67^{cd}
7	3.93±0.70^{abcd}	8.14±0.79^{a}	6.82±0.84^{a}	1.32±0.25^{a}	3.36±0.60^{a}	1.43±0.15^{a}	3.85±0.32^{ab}	2.61±0.26^{ab}	14.23±2.84^{abcd}	3.32±0.35^{a}	14.74±1.51^{a}
8	4.00±0.71^{abcd}	32.89±4.48^{bc}	28.31±4.49^{bcd}	4.69±1.39^{a}	7.00±1.35^{ab}	2.62±0.28^{abc}	6.17±0.60^{bc}	3.44±0.45^{abc}	26.08±3.07^{abcd}	3.86±0.67^{ab}	19.50±1.66^{abc}
9	3.43±0.81^{abcd}	15.07±3.70^{ab}	14.00±3.55^{abc}	1.07±0.32^{a}	4.93±0.72^{ab}	1.56±0.17^{ab}	2.99±0.36^{a}	1.83±0.19^{a}	6.67±1.49^{ab}	3.45±0.41^{a}	17.10±2.59^{ab}
10	1.00±0.00^{a}	9.39±2.17^{a}	8.45±2.05^{ab}	1.43±0.26^{a}	7.29±0.85^{ab}	0.97±0.12^{a}	2.45±0.29^{a}	1.91±0.19^{ab}	4.25±0.53^{a}	3.01±0.26^{a}	25.36±4.02^{abcd}
11	2.86±0.65^{abc}	37.68±8.52^{cd}	30.66±7.01^{cd}	7.02±3.38^{ab}	7.50±2.45^{ab}	3.68±0.62^{c}	8.21±1.07^{cde}	4.29±0.73^{bcde}	31.59±7.01^{bcde}	3.31±1.32^{a}	19.21±3.63^{abc}

[a] Mean data are expressed with the standard error of the mean±SE. Means of the same populations not sharing the same letters are significantly different (Tukey HSD,$P < 0.05$). All traits show significant differences between populations $P < 0.001$ (One-Way ANOVA). Values highlighted in **bold** correspond to the highest value for the morphological trait, and underlined values correspond to the lowest value measured.
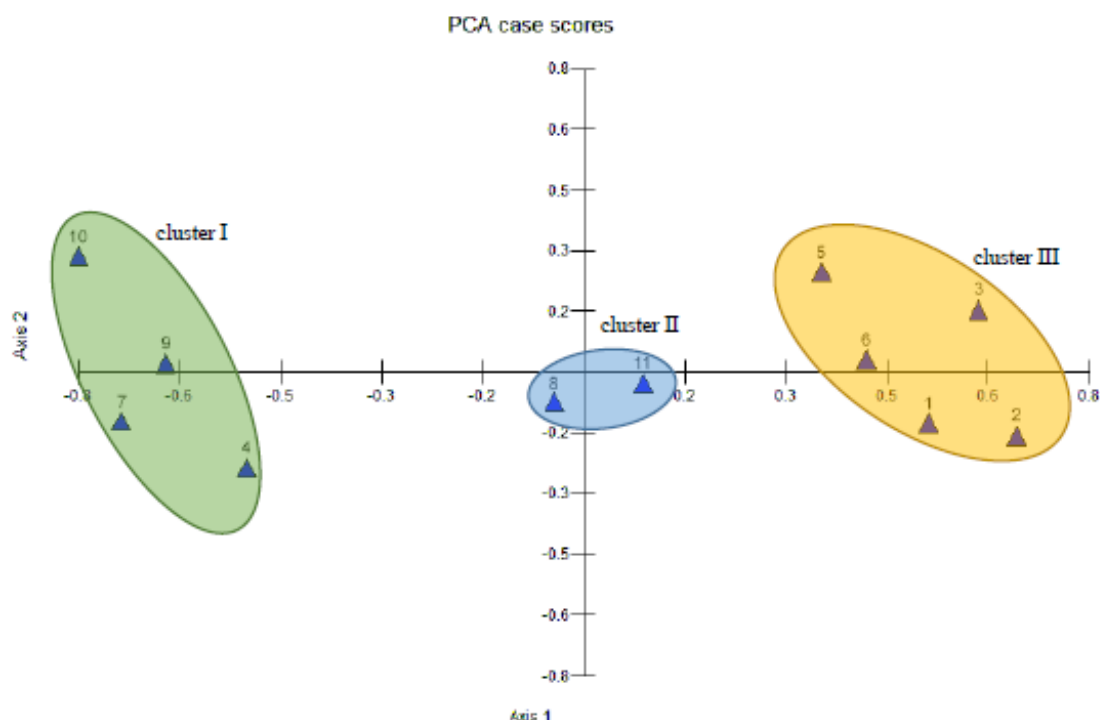
Figure 2. PCA for morphological characterization of *B. vulgaris* subsp. *maritima* populations. The first 2 components explain 93.3% of observed field variation (axis 1 explaining 85.2% and axis 2 explaining 8.1% of total variability).

Table 4. Morphological traits' average differences, represented by ANOVA for *B. vulgaris* subsp. *maritima* populations, based on the discriminant analysis clustering.[a]

	Cluster I	Cluster II	Cluster III
Number of populations per cluster	4	2	5
Number of Basal Stems (NBS)	4.05 ± 2.84^a	3.43 ± 0.81^a	4.41 ± 3.28^a
Plant Height (PH)[**]	11.46 ± 3.24^a	35.29 ± 3.38^b	68.17 ± 8.53^c
Inflorescence Height (IH)[**]	9.88 ± 3.08^a	29.48 ± 1.66^b	50.10 ± 5.76^c
Distance of the first Branch from the Basis (DBB)[‡]	1.70 ± 0.86^a	5.86 ± 1.65^a	18.07 ± 3.78^b
Number of Branches (NB)[*]	4.91 ± 1.71^a	7.25 ± 0.35^a	16.06 ± 4.30^b
Leaf Width (LW)[**]	1.39 ± 0.29^a	3.15 ± 0.75^{ab}	4.55 ± 1.04^b
Leaf Length (LL)[*]	3.29 ± 0.69^a	7.19 ± 1.44^b	8.72 ± 1.93^b
Leaf Length/Leaf Width (LL/LW)	2.41 ± 0.39^a	2.33 ± 0.14^a	2.02 ± 0.17^a
Petiole Width (PW)[**]	2.18 ± 0.37^a	3.86 ± 0.60^{ab}	5.56 ± 0.97^b
Petiole Length (PL)[*]	9.45 ± 4.75^a	28.83 ± 3.89^b	38.76 ± 10.04^b
Stem Diameter (SD)[**]	8.61 ± 1.71^b	3.59 ± 0.39^a	3.19 ± 0.23^a
Average Number of Glomerulus per branch (NG)[*]	30.73 ± 3.08^b	19.36 ± 0.20^a	18.58 ± 4.65^a

[a] Morphological traits' data are expressed in mean ± SD. Means of the same cluster not sharing the same letters are significantly different (Tukey HSD, P< 0.05). Traits showing significant differences between clusters are labeled as follows [**]< 0.001, [*]< 0.05 (One-Way ANOVA).

Madeira Island. The most significant differences occur between clusters I and III (F= 173.877) followed by the differences between clusters III and II (F= 42.289). Clusters I and II present higher similarity (F= 16.707). There is a clear separation between populations from the eastern part of Madeira Island (POPs 4 and 7) and Porto Santo (POPs 8, 9, 10, and 11) and populations from western (POPs 1 and 2) and southern parts of Madeira Island (POPs 3, 5, and 6).

The traits that contributed the most to the segregation of *B. vulgaris* subsp. *maritima* populations are PH and IH. These two traits clearly divide all three clusters using the Tukey Test (P< 0.001). DBB, LW, PW, and SD traits also show high differences (P< 0.001) but did not contribute as much to separate all three clusters as PH and IH did. Traits NB, LL, PL, and NG show lower values of F (P< 0.05) but are still significant. NBS and LL/LW ratio do not influence the separation of the clusters.

Molecular Analysis

A total of 77 alleles were detected, with a maximum of 15 alleles for 2KWS and a minimum of 7 alleles for FDSB1027, FDSB1250, and SB13 each, with a mean of 9.6 alleles per molecular marker. One allele was considered null, as it did not amplify.

Shannon-Wiener's Index (H') for markers *loci* polymorphism, has average values ranging from 0.856 (POP 10) to 1.381 (POP 6) (Table 5). The most genetically diverse

population is POP 6 (Madeira Island), followed closely by POP 8 (Porto Santo). POP 10 (Porto Santo) is the least diverse, followed by POPs 7 and 4 (both located in Ponta de São Lourenço, Madeira, Figure 1). Levene's observed heterozygosity (Table 5) has low values overall, varying from 0.400 (POPs 1, 2, and 7) to 0.650 (POP 9) and it is always lower than the expected heterozygosity, which ranges from 0.575 (POP 10) to 0.787 (POP 6). For Nei's expected heterozygosity, populations follow the same order (minimum to maximum) as in Levene's results. Average heterozygosity (all populations) is 0.635 (data not shown), meaning that there is a moderate proportion of heterozygous individuals. For the Total Allele Number (TAN; different alleles within a population) per SSR marker, the highest value is 38 (POP 6) and the lowest is 22 (POP 10).

PIC values vary between 0.8852 (2KWS) and 0.7168 (FDSB1250) (data not shown), with a mean value of 0.808 (results all above 0.7), which indicates that these markers are good diversity indicators (Botstein *et al.*, 1980; Abbasi *et al.*, 2014). According to F_{IS} calculations, markers that present observed heterozygosity excess are SB13, BQ584037, and BQ588629 (-0.092, -0.126 and -0.423, respectively), and observed heterozygosity deficiency are SB15, FDSB1027, 2KWS, FDSB1250 and SB04 (0.622, 0.432, 0.337, 0.333, and 0.107, respectively), with an average value of 0.186.

For populations from Madeira Island, POP 6 (southern Madeira Island) stands out as displaying the highest values in all

Table 5. Summary table of molecular analysis for *B. vulgaris* subsp. *maritima* populations.[a]

Population	1	2	3	4	5	6	7	8	9	10	11
H'	1.255	1.130	1.187	1.026	1.216	1.381	0.983	1.327	1.187	0.856	1.268
Obs. Het.	0.400	0.400	0.525	0.575	0.550	0.588	0.400	0.525	0.650	0.525	0.550
Exp. Het.	0.722	0.700	0.717	0.650	0.728	0.787	0.644	0.764	0.731	0.575	0.747
Nei's Exp. Het.	0.650	0.630	0.645	0.585	0.655	0.706	0.580	0.688	0.658	0.518	0.673
TAN	34	30	32	26	32	38	25	36	30	22	33

[a] H' mean values, Observed and Expected Heterozygosity, and Nei's Expected Heterozygosity (Obs. Het., Exp. Het. and Nei's Exp. Het., respectively).

parameters. Contrarily to populations from the eastern part of Madeira, Ponta de São Lourenço populations (4 and 7) seem to be less diverse, as they have the lowest values overall (except POP 4 for Obs. Het). For Porto Santo's populations, heterogeneity between populations is observed. There is a substantial difference between POPs 8 (TAN = 36) and 10 (TAN = 22). POP 8 is the most genetically diverse, with the second-highest presence of alleles overall (36) and POP 10 displays the lowest values for all genetic parameters (except for Obs. Het, which is equal to POP 8), making this population as the less diverse of all eleven populations analyzed. The genetic analysis of beet populations shows that the molecular markers 2KWS and SB15 linked with ECS, SC, and WSC traits in saline and non-saline conditions, and sugar yield-related traits and response to salinity, respectively (Table 2), have high diversity, showing a total of 49 and 43 alleles (data not shown). In the case of 2KWS, 5 unique alleles were detected among beet populations, and 3 of them were detected in POP 9.

PCoA (Figure 3) shows that populations from Madeira and Porto Santo differentiate between themselves, as populations from Madeira appear in the upper and lower right quadrants and populations from Porto Santo appear in the upper and lower left quadrants.

From a genetic diversity point of view, POP 7 occupies an intermediate distance between Porto Santo and Madeira populations. Populations from Madeira appear to disperse and, clearly, clustering based on their geographical distribution cannot be achieved, which agrees with its isolation. Looking at Porto Santo's populations, POP 9 is genetically different from the other 3 populations (POPs 8, 10, and 11) and can be grouped in a single cluster.

DISCUSSION

Results for morphological analysis show that there is a clear gradient for all measured traits, with higher values decreasing from the west to the east of Madeira Island and continuing to Porto Santo to the transition of plant habit, from erect to prostrate. Cluster III populations show higher values of NB, LW, and LL, traits of interest for leaf beets. These populations occur over cliffs, in less exposed sites, and show a tendency to have bigger and more developed aerial parts and different seed production strategies. Opposite to this, clusters I and II populations occur closer to the sea, exposed to wind, under dry and saline conditions, resulting in plants that are prostrated, with a smaller leaf area to reduce evapotranspiration and higher
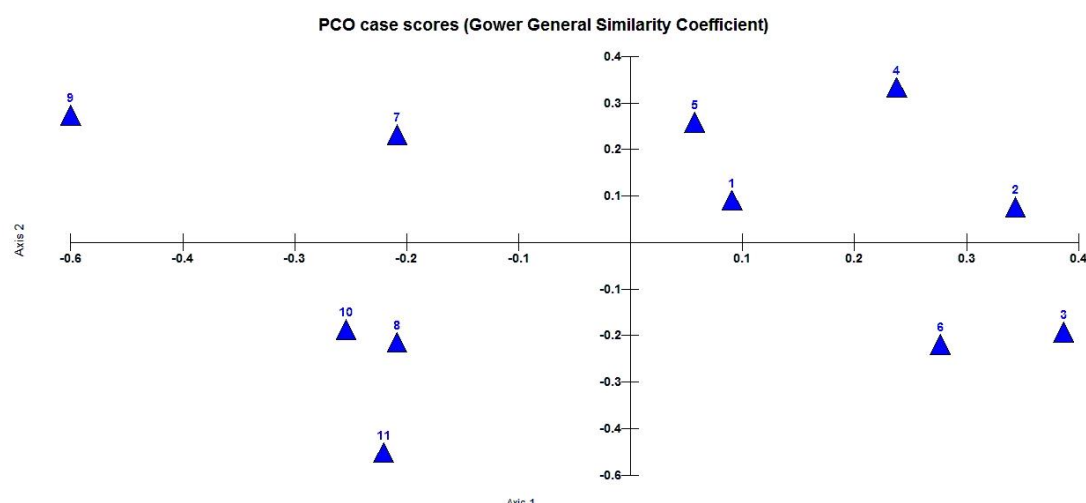
Figure 3. PCoA distribution of *B. vulgaris* subsp. *maritima* populations, according to genotypic data.

investment in seed production. Our raw populations' size estimations point out that all eleven populations have a low effective number, ranging from few tens to a maximum of hundred individuals, with many isolated plants.

Sea beet is an allogamous species (cross-pollinated), wind-pollinated, and has a gametophytic self-incompatibility system that prevents self-pollination (Panella *et al.,* 2007), allowing the possibility of cross-pollination with beet crops (Pinheiro de Carvalho *et al.*, 2010; Castro *et al.*, 2013) that exist in areas with high human presence, where traditional cultivation of leaf beets occurs. This is the case for cluster III populations, in contrast to clusters I and II populations, which are located either on protected areas, such as the PSL 2000 Network area or in remote areas of Porto Santo. POPs 4 and 7 present in cluster I share the habitat with a beet endemic species, *B. patula* (Pinheiro de Carvalho *et al*., 2012; Frese *et al.*, 2019), resulting in the hypothesis of a limited cross-pollination between *B. patula* and *B. vulgaris* subsp. *maritima* that could be removing alleles from the latter gene pool, resulting in less variability (Biancardi *et al.*, 2012a). This would explain their clustering with Porto Santo´s most isolated populations in cluster I. Adding overall lowest results for the H' and TAN, these conclusions are reinforced.

Considering TAN values and combining them with the three PCA clusters, we can notice that populations from cluster II have the highest TAN values among all populations from Porto Santo, and whose values are more similar to Madeiran populations (cluster III). Therefore, it appears that these populations are closer to this cluster and far from the cluster I populations, where two Madeiran and two Porto Santo populations appear grouped, having the four lowest values of TAN among all eleven populations.
Analyzing the PCoA, it is evident that sea beet populations from Madeira are genetically different than populations from Porto Santo, showing high genetic

variability between populations, as they differ according to their geographical origin. Populations from Madeira Island are dispersed in the upper and lower right in the PCoA distribution (Figure 3). Apart is POP 7 that is closer to the Porto Santo cluster, and which habitat constraints, followed by its geographical position, present a great similarity to Porto Santo characteristics. As shown in the PCA, POP 4, like POP 7, are under influence of a similar habitat. We hypothesize that POP 4 is not genetically different from the rest of Madeiran populations as shown in the PCoA, since it is not isolated by a geographic barrier as in the case of POP 7, which occurs in Desembarcadouro islet and suffers from genetic drift affecting small populations. Therefore, POP 4 shares more genetic similarities with Madeira´s populations, and POP 7 with Porto Santo populations, but these assumptions need further studies and specific analyses that are not in the context of this study.

Madeiran populations that are geographically close to each other do not aggregate in PCoA, giving no evidence of a significant trend in genetic segregation between the island populations. For Porto Santo, POP 9 distinguishes itself from the other three populations, which might be an indication that the habitat where POP 9 occurs (no human disturbance, 7 m from sea level, exclusively rocky substrate – different from every other population in this study) has selected a more specific genotype with better adaptation to the environmental specific conditions. For example, Abbasi *et al.* (2014) observed that the heritability estimates in sugar beet were smaller in saline soils. In this study, some of our molecular markers are linked to traits related to responses to saline conditions and developmental processes in plants. These traits result from a combination of multiple genes that are influenced by environmental interactions (Arzani, 2008). This leads to the possibility that environmental constraints are stronger influencers of populations' genetic variability than the gene flow between our

sea beet populations, otherwise, POP 7 would have to be clustered with the rest of the Madeira populations. There are genetic differences comparing the two islands, but not enough in the same island that could explain differences shown by morphological analysis, also implying that our morphological results could be a response to adaptations based on epigenetic factors (Arzani and Ashraf, 2016) since there is more morphological variation than genetic variation in sea beet populations from the archipelago.

Although F_{IS} values vary greatly, with an average value of 0.186, it indicates that, overall, populations were not under inbreeding or bottleneck events. These results make available additional information about sea beet genetic resources in Madeira's archipelago and help to understand their importance as additional sources of genetic material for crop breeding. However, there are still improvements to make regarding the use of marker-assisted selection for breeding purposes that still rely much on the phenotypic selection (Arzani and Ashraf, 2016). The genetic analysis of beet populations via SSRs seems to support our thoughts that environmental conditions are the driver in the enhancement of observed diversity.

CONCLUSIONS

This work allowed us to gather new information from *B. vulgaris* subsp. *maritima* populations from Madeira archipelago. When analyzing morphological traits, populations were grouped into three clusters. There is a clear separation of populations from: (1) Western and southern parts of Madeira, (2) Two intermediate populations of Porto Santo, and (3) To those of the eastern part of Madeira and Porto Santo. Results from the genetic characterization show that diversity is related to geographic distribution. There seems to be a link between morphological

and genetic traits. The less genetically (H' and TAN) diverse populations were part of the same cluster (I) that grouped plants with smaller sizes. Populations with intermediate and highest genetic diversity were grouped in clusters II and III, which included plants with bigger sizes. Further studies should be made to improve the knowledge about these populations. More markers should be used and linked to morphological traits, more individuals should be sampled in each population, and new populations should be included from other sites around Madeira's archipelago such as the Desertas and Selvagens Islands.

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برآورد تنوع در جمعیت گیاه چغندر دریایی (*Beta vulgaris* L. ssp. *maritima*)

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چکیده

چغندر دریایی (Arcang (L.) *Maritime* ssp. *Beta vulgaris*) گونه ای است با تنوع های ژنتیکی و مورفولوژیکی که به خزانه ژنی اولیه چغندر تعلق دارد. این خویشاوند وحشی گیاه یک منبع

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ارزشمند ژنتیکی برای بهبود مقاومت در چغندر است و میتواند در پایداری عملکرد گیاه نقش مهمی داشته باشد. در این پژوهش، ۱۱ جمعیت چغندر دریایی از منطقه دریای مادیران (Madeiran) با استفاده از توصیف گرهای مورفولوژیکی و نشانگرهای ژنتیکی مورد تشخیص قرار گرفت. هدف آزمایش ارزیابی این جمعیت ها به عنوان منبع مستعدی از مواد ارزشمند ژنتیکی بود. تشخیص مورفولوژیکی، تغییرات کمّی زیادی میان جمعیت های مزبور نشان داد. پارامترهای طول گیاه و گل آذین بیشترین تاثیر را در جدا سازی جمعیت ها داشتند. برای تعیین تغییرات ژنتیکی بین جمعیت ها، تجزیه ملکولی با استفاده از SSRهای چند شکلی(پولی مورفیک) انجام شد. هر دوی PCA و PCoA سه خوشه را آشکار ساختند که جمعیتهای مزبور را به ترتیب بر حسب صفات مورفولوژیکی و ژنتیکی جدا سازی میکرد. نتایج این پژوهش به دانستنی های مربوط به تنوع چغندر دریایی در مجمع الجزایر Madeira و نیز به این استنباط که شرایط محیطی خاص این جزایر تنوع ژنتیکی آن را تحت تاثیر قرار میدهد کمک کرده و این جمعیت ها را به عنوان یک منبع ژنتیکی ممکن برای برنامه های بهنژادی چغندر قند مهیا میسازد.