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Original
Using simple sequence repeats in 9 brassica complex species to assess hypertrophic curd induction / Treccarichi, S., Di Gaetano, C., Di Stefano, F., Gasparini, M., Branca, F.. - In: AGRICULTURE. - ISSN 2077-0472. - 11:7(2021), p. 622. [10.3390/agriculture11070622]

Availability:
This version is available at: 11583/2917718 since: 2021-08-12T10:37:46Z

Publisher:
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Published
DOI:10.3390/agriculture11070622

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01 November 2026

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Using Simple Sequence Repeats in 9 *Brassica* Complex Species to Assess Hypertrophic Curd Induction

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Abstract: Five Simple Sequence Repeats (SSRs) were used to assess the relationship between inflorescence characteristics and their allelic variation in 53 *Brassica oleracea* and *Brassica* wild relatives (n = 9). Curd morphometric traits, such as weight (CW), height (CH), diameter (CD1), shape (CS) inflorescence curvature angle (CA), and its curd stem diameter (CD2), were measured. The aim of the work was to analyze the relationships among the allelic patterns of the SSRs primers utilized, and their status of homo or heterozygosity registered at each locus, as well as the inflorescence morphometric traits in order to individuate genomic regions stimulating the hypertrophy of this reproductive organ. The relationships found explain the diversity among *B. oleracea* complex species (n = 9) for the inflorescence size and structure, allowing important time reduction during the breeding process by crossing wild species, transferring useful resistance, and organoleptic and nutraceutical traits. The five SSRs loci were BoABI1, BoAP1, BoPLD1, BoTHL1, and PBCGSSRBo39. According to the allelic variation ascertained, we evaluated the heterozygosity index (H) for each SSR above cited. The results showed a significant interaction between the H index of the BoPLD1 gene and the inflorescence characteristics, summarized by the First Principal Component (PC1) (p-value = 0.0244); we ascertained a negative correlation between the H index and inflorescence characteristics, namely CW, CH, CD1, CD2, CA. The homozygosity BoPLD1 alelles, indicated by the H index, affect the inflorescence characteristics and broccoli and cauliflower yields.

Keywords: *Brassica* complex species; MADH–box genes; SSRs assay; heterozygosity index; allelic variance; curd morphometric traits

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Citation: Treccarichi, S.; Di Gaetano, C.; Di Stefano, F.; Gasparini, M.; Branca, F. Using Simple Sequence Repeats in 9 *Brassica* Complex Species to Assess Hypertrophic Curd Induction. *Agriculture* **2021**, *11*, 622. https://doi.org/10.3390/agriculture11070622

Academic Editor: Rodomiro Ortiz

Received: 31 March 2021
Accepted: 28 June 2021
Published: 1 July 2021

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1. Introduction

Brassica crops include several interesting species which are strictly related to crop wild relatives (CWRs) during their domestication process [1]. The Mediterranean region represents one of the main domestication and diversification centers of *Brassica* genus, in particular in Sicily where the cytodeme is represented by several wild relatives such as *Brassica macrocarpa* Guss., *B. villosa* Biv., B. *rupestris*, and B. *incana* [2].

The brassica genus includes three diploids (2n) (AA, BB, CC) and three tetraploids (4n) (AABB, AACC, BBCC) main species as described in the U's triangle model [3]. The *B. oleracea* complex species (n = 9) belongs to genome C (n = 9) and it represents the primary gene pool of the *Brassica* genus. This genus shows high genetic variability due to the genetic self-incompatibility characterizing the landraces and their CWRs and to several domestication processes [4]. Genetic diversity of *B. oleracea* is shown by the several varieties obtained by different domestication processes in a number of geographic areas which include broccoli, cauliflower, cabbage, kale, kohlrabi, savoy cabbage, and Brussel sprouts.

Brassica wild relatives could be a source of cytoplasmic male sterility (androsterility) for the development of hybrid seed of *Brassica* crops and they can provide genes for

resistance to different diseases and pests and for these traits they can be used in breeding programs [5].

Cauliflower and broccoli are characterized by the floral induction of hypertrophic inflorescence [6]. Broccoli crop, as reported by Viani, originated from wild cabbage while cauliflower head derived from the improving process of broccoli addressed to the reduction of branches length, flower bud size, and the absence of their pigmentation.

Flower development genes were studied by Bowman et al. who found several genes involved such as *apetala 1* and *cauliflower* in *Arabidopsis* [7]. These genes are closely related to members of the MADH-box genes family and a mutant copy of them is present in *B. oleracea* genome. Irish and Sussex characterized several floral phenotypes produced by the recessive homeotic *apetala 1* (*ap1*) mutation in *Arabidopsis* and the homozygote for this mutation showed weak inflorescence affecting floral primordia formation [8].

Smith and King proposed a simple genetic model based on segregation of recessive alleles for BoAP1 and BoCAL candidate genes which showed differences in stage of arrest between cauliflower and Calabrese broccoli [9]. According to Smith and King's allelic distribution genetic model, the domestication process reduced the allelic diversity by promoting loci affecting the arrest of floral development which determined the inflorescence hypertrophy and then the domestication for cauliflower's curd phenotype; the Sicilian Purple was indicated as an important intermediate of this domestication process [10].

BoABI1, BoAP1, BoPLD1, and BoTHL1were designed to amplify genomic DNA region by Tonguc and Griffiths [11]. They were used to assess genetic similarity between several *B. oleracea* cultivars, belonging to three varietal groups (cabbage, cauliflower, and broccoli) while PBCGSSRBo39 was designed by Burgess et al. [12] to provide a useful molecular marker for crop improvement which was derived from shotgun sequencing programs.

Simple sequence repeats markers can be a useful tool to find genetic relationships among genotypes and related species provided from different countries. They can be used also as chloroplastic SSRs (cpSSRs) to avoid multiple gene copy number problems in polyploidy species [13].

In this study, the inflorescence morphometric traits of several accessions of broccoli and cauliflower landraces and commercial varieties, and *Brassica* relatives were measured [14], and additionally the five SSRs above cited were utilized to analyze the allelic variation among the accessions used and to associate them with inflorescence characteristics.

2. Materials and Methods

Plant material was represented by fifty-three accessions belonging to the Department of Agriculture, Food and Environment (Di3A) of the University of Catania-UNICT (Table 1). Seeds were sown in the first week of July in cellular trays placed under greenhouse conditions. The seedlings were transplanted after 5 weeks on the experimental farm of the University of Catania, (37°27′ N, 15°40′ E, 10 m a.s.l.) in single rows, with 1.0 m between the rows and 0.5 m between the plants along the rows, at crop density of 2 plants/m^2. The experimental design was composed of four replicates (10 plants each) placed in randomized blocks as described by Branca et al. [15]; plants were grown in open fields.

For the accessions, inflorescence morphological data were registered following the International Board for Plant Genetic Resources (IBPGR) [16] descriptors related to the curd. Inflorescence morpho-biometric traits such as weight (CW), height (CH), diameter (CD1), shape (CS), angle of curvature (CA), and inflorescence stem thickness (CD2) were measured and calculated at the laboratory of Biotechnology of Vegetable and Flower Crops of the Di3A UNICT department. The inflorescence before anthesis was cut five centimeters before the first branch of inflorescence and for it the CW was registered by analytical balance, the CH and CD1 were calculated using a meter rule while CD2 was calculated using a caliber. The inflorescence shape (CS) parameter can be used to distinguish broccoli and cauliflowers from the CWRs and is derived from the ratio between CH and CD1. Curvature angle CA was registered with a goniometer by calculating the angle limited to between the central vertical inflorescence axes and the tangent to the extreme part of it.

Table 1. List of *B. oleracea* complex species (*n* = 9) accessions utilized.

Accession Code	Laboratory Code	Origin	Species
UNICT 3876	CV 171 Menhir F1	ISI sementi	CV
UNICT 3190	BR 15 S 1 A	Modica (RG)	CV
UNICT 4137	CV 99 S2 B	Adrano (CT)	CV
UNICT 4145	BR 13 S3 AC	Modica (RG)	CV
UNICT 3878	CV 173 Freedom	3878 Royal Sluis	CV
UNICT 4138	CV 76 S2	Acireale (CT)	CV
UNICT 3652	CV 159	Catania	CV
UNICT 3900	BR 13 A X CV98/21	DISPA 4	CV
UNICT 3902	CV 33 S1	Royal Sluis	CV
UNICT 3895	CV 98/2 X CV 136 EG	DISPA 2	CV
UNICT 3880	CV 175 White Flash	Sakata	CV
UNICT 3879	CV 174 Graffiti	ISI sementi	CV
UNICT 3089	CV 75 S3AC	Acireale (CT)	CV
UNICT 3906	CV 24 S4	Biancavilla (CT)	CV
UNICT 3892	CV 98/2 X BR 13 S3	DISPA 3	CV
UNICT 579	BR 41	Modica (RG)	CV
UNICT 3578	BR 165 Marathon	Esasem	BR
UNICT 3893	CV 136 EG X CV98/2	DISPA 1	CV
UNICT 3671	CV 72 S2	Catania (CT)	CV
UNICT 583	BR 46	Vittoria (RG)	BR
UNICT 658	BR 45 S1	Acireale (CT)	BR
UNICT 3669	BR 17 S2	Ragusa (RG)	CV
UNICT 658	BR 129	Roccella Valdemone (ME)	BR
UNICT 657	BR 128	Roccella Valdemone (ME)	BR
UNICT 651	BR 122 Packman	Petoseed	BR
UNICT 655	BR 126	Adrano (CT)	BR
UNICT 3674	CV 19 S2 A	Piazza Armerina (EN)	CV
UNICT 637	BR 106	Cefalù (PA)	BR
UNICT 3675	BR 94 S1	Francavilla (ME)	BR
UNICT 3668	BR 115 S1	Troina (EN)	BR
UNICT 574	BR 36	Biancavilla (CT)	BR
UNICT 342	*Brassica macrocarpa 1*	Favignana (TP)	BM
UNICT 733	*Brassica rupestris 1*	San Vito Lo Capo (TP)	BU
UNICT 342	*Brassica macrocarpa 2*	Favignana (TP)	BM
UNICT 342	*Brassica macrocarpa 3*	Favignana (TP)	BM
UNICT 3512	*Brassica incana 1*	Agnone Bagni (SR)	BY
UNICT 3270	*Brassica rupestris 2*	Bivongi (RC)	BU
UNICT 3270	*Brassica rupestris 3*	Bivongi (RC)	BU
UNICT 342	*Brassica macrocarpa 4*	Favignana (TP)	BM
UNICT 3512	*Brassica incana 2*	Agnone Bagni (SR)	BY
UNICT 342	*Brassica macrocarpa 5*	Favignana (TP)	BM
UNICT 732	*Brassica rupestris 4*	Roccella Valdemone (ME)	BU
UNICT 732	*Brassica rupestris 2*	Roccella Valdemone (ME)	BU
UNICT 342	*Brassica macrocarpa 6*	Favignana (TP)	BM
UNICT 736	*Brassica rupestris 5*	Ragusa Ibla (RG)	BU
UNICT 342	*Brassica macrocarpa 7*	Favignana (TP)	BM
UNICT 4158	*Brassica incana 3*	Sortino (SR)	BY
UNICT 736	*Brassica rupestris 6*	Ragusa Ibla (RG)	BU
UNICT 3040	*Brassica villosa 1*	Marianopoli (CL)	BV
UNICT 736	*Brassica rupestris 7*	Ragusa Ibla (RG)	BU
UNICT 342	*Brassica macrocarpa 8*	Favignana (TP)	BM
UNICT 4158	*Brassica incana 4*	Sortino (SR)	BY
UNICT 3040	*Brassica villosa 2*	Marianopoli (CL)	BV

Legend: CV—Cauliflower; BR—Broccoli; BY—*B. incana*; BM—*B. macrocarpa*; BU—*B. rupestris*; BV—*B. villosa.*

For morphological data, the mean values of the analyzed parameters of every accession were used to prepare a numerical matrix.

Genomic DNA was extracted from seedlings upon reaching the 6–8 leaved stage in young leaves tissues as reported by Tonguç and Griffiths utilizing the kit GenEluteTM Plant Genomic DNA Miniprep (Sigma Aldrich Inc.).

Extracted DNA was measured using a spectrophotometer Shimadzu at wavelengths of 260 and 280 nm, quantified by visual comparison on ethidium bromide-stained agarose gels. The final DNA concentration followed the protocol established by Branca et al. (2018) which includes 200 ng of template DNA.

The primers flanking SSR sequences (Table 2) were obtained in accordance with Tonguc and Griffiths (2004) for BoTHL1, BoAP1, BoPLD1, and BoABI1; concerning the PBCGSSRBO39 primers sequence, this was retrieved by Burgess et al. The position of the primers was checked using Assembly: GCA_0006955251.1 within Ensembl.

Table 2. List of primers utilized with their sequences and chromosome position.

GenBank	Primers Name	SSR Motif	Primer Sequence (Forward, Reverse)	Chromosome
AF113918	BoPLD1	$(CT)_7(AT)_{7-1}$	GACCACCGACTCCGATCTC AGACAAGCAAAATGCAAGGAA	C5
AF180355	BoABI1	$(TC)_{16}$	TATCAGGGTTTCCTGGGTTG GTGAACAAGAAGAAAAGAGAGCC	C1
AF273844	BoTHL1	$(CTT)_7$	GCCAAGGAGGAAATCGAAG AAGTGTCAATAAGGCAACAAGG	C9
U67451	BoAP1	$(AT)_{9-1}$	GGAGGAACGACCTTGATT GCCAAAATATACTATGCGTCT	C6
BH479680	PBCGSSRBo39	$[GGTCG]_4$	AACGCATCCATCCTCACTTC TAAACCAGCTCGTTCGGTTC	C7

Five SSRs primers used were chosen following Branca et al., selecting them from ten primers, performed by Branca et al. for phylogenetic analysis and to assess the genetic similarity between several *B. oleracea* cultivars and wild Brassica species, belonging to two varietal groups (cauliflower and broccoli) as well as to estimate genetic divergence using F_{ST} statistic; broccoli cultivars clustered with cauliflower cultivars as predicted and wild species showed major genetic differences [13].

The basic local alignment search tool (BLAST) was performed to check amplicon size and to compare results with amplificated sequences registered in an online database which was represented by BLAST (version 1.17) and Ensembl. The Uniprot database (release 2021, version 3) was used to study encoding regions close to the gene of interest.

The SSRs studied are located in different regions of the plant genome: BoABI1 is located in chromosome 1 region: 1,229,915,511-12,992,170 within the gene Bo1g041870 coding the ABI1 protein. The second SSR BoTHL1 is located on chromosome: 17,254,558: 17,255,176 within the Bo9g058820 gene, a homolog of thioredoxin 3 in Arabidopsis thaliana. The microsatellite PBCGSSRBo39 is located inside the Bo7g105720 gene on chromosome 7, BoAP1 is located inside chromosome 6: 33,883,667-33,887,357 inside the Bo6g108600 gene, one of MADS-box gene family members (Ap1Like).

BoPLD1 marker is located in the fifth chromosome in *B. oleracea* from 46,037,340 bp to 46,037,606 bp.

After DNA purification, PCR-based amplification was performed in 20 µL of final volume. The reaction mixture was composed of 200 ng of DNA template, 200 µM of each dNTP 3.75 mM MgCl2, 1X Taq DNA polymerase buffer, and 2 mM Primer according to Branca et al. (2018). DNA amplification was conducted in a Perkin Elmer 9700 thermocycler (ABI, Foster City, CA, USA) with the following parameters: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. At the end of reaction, amplicons were stored at 4 °C. PCR products were loaded into 4% agarose gels (UNILAB Life Science, Taipei, Taiwan) and the electrophoresis run at a

voltage of 100 V for 5–6 h in 1 X TBE buffer [15]. Capillary electrophoresis was performed using ABI PRISM 3130 Genetic 191 Analyser (Applied Biosystems, Waltham, MA, USA) as described by Branca et al. (2013) and Branca et al. Fragment sizes were determined by the GeneMapper 3.7 software (Applied Biosystems, Waltham, MA, USA). The allele peaks were checked by performing capillary electrophoresis and also checked using GeneMapper software. Each allele peak was manually rechecked by the operator.

3. Data Analysis

Allelic detection occurred in coding allelic status on the basis of their molecular weights using numeric scores: 2 (homozygosity), 1 (heterozygosity), and 0 (absence of any allele).

Allelic frequency data were elaborated to calculate heterozygosity index (H) which indicates the frequencies of heterozygosity in a population; an H value close to 1 suggests a large degree of heterozygosity within the populations while an H value close to 0 suggests homozygosity. Statistical analysis was performed to evaluate the correlation between the heterozygosity index for each locus and the inflorescence morpho-biometric traits as CW, CD1, CD2, and PC1.

For statistical analysis, the main inflorescence morpho-biometric characteristics, with exception of CS were used to calculate the Principal Component Analysis (PCA) to obtain a single parameter to summarize the inflorescence characteristics. CS was discarded due to the origin of this parameter which is derived from CH and CD1. PCA was performed using RStudio software 3.6.3 and a linear regression model was used to obtain information about the relationships between the heterozygosity index of each locus and the inflorescence bio-morphometric traits. PCA data were scaled to have unit values.

4. Results

Inflorescence morphometric traits CW, CH, CD1, CD2, and angle of curvature CA were registered for the Di3A accessions establishing a morphological database (Table 3).

The inflorescence morphometric variance was detected using data on plant bio-morphometric parameters recorded on Sicilian broccoli and cauliflower landraces and their F1 hybrids which show a large diversity among the genotypes.

The curd weight showed higher values for cauliflower landraces and F1 hybrids, than the CWRs analyzed, which registered lower values. Among cauliflower accessions, CV 171 Menhir F1 recorded the highest value (10,958 g); landrace curds weighed less than those collected from F1 hybrids and that explain the worldwide diffusion of these genotypes due to their yield. Curd diameter (CD1) was related to CW and, as registered, showed lower values in CWRs accessions while cultivated accessions with higher CD1 values, such as CV 99 S2 B recorded the largest curd diameter, respectively 21.1 cm (Table 3).

Broccoli types showed an elongated inflorescence as shown by CS values while cauliflowers showed more compact and flattened curd such as compared to the CWRs. Broccoli landraces showed the highest CS values compared to commercial cultivars.

The curvature angle (CA) also showed the large phenotypical variability among broccoli and cauliflowers landraces and hybrids F1, and their CWRs. CA distinguish well cauliflowers from broccoli and CWRs; cauliflowers were characterized by the highest CA value. Broccoli accessions showed lower CA values than cauliflowers. CWRs were characterized by the absence of the hypertrophic inflorescence developed from the apical meristem and they showed the lowest CA values; *B. macrocarpa* accession Favignana 1 showed the lowest CA value (7°).

Phenotypical variability was explained using a correlation model for each bio-morpho-metric descriptor; PC1 showed 46.75% of the total variance among the accessions (Table 4) and it is significantly correlated to CW, CD1, CD2 and CA. PC2 overlaps with CH which is one of the major traits affecting inflorescence morphology and therefore it was not used for this analysis.

Table 3. Inflorescence morphometric characteristic in descending order, from the heaviest to the lightest. The parameters measured were curd weight (CW), height (H), curvature angle (CA), curd and stem diameters (CD1 and CD2) and principal component 1 (PC1).

Laboratory Code	CW (g)	CH (cm)	CD2 (cm)	CD1 (cm)	CS (cm)	CA (°)	PC1
CV 171 Menhir F1	1095.8 (21.1)	11.1 (8.4)	42.32 (8.5)	18 (8.7)	0.62 (9.6)	110 (21.9)	3.794
BR 15 S 1 A	965.7 (37.4)	15.4 (14.6)	39.82 (16.4)	20.7 (17.4)	0.74 (16.6)	105 (19.4)	3.588
CV 99 S2 B	666.6 (42.5)	15.2 (13.2)	34.09 (19.6)	21.1 (15.0)	0.72 (12.1)	112 (20.4)	2.925
BR 13 S3 AC	628.8 (33.7)	16.8 (16.6)	38.13 (18.5)	19.7 (14.6)	0.85 (14.6)	101 (22.5)	2.742
CV 173 Freedom	605 (33.8)	89 (16.7)	30.99 (10.3)	16.9 (11.8)	0.53 (12.1)	113 (13.3)	2.171
CV 76 S2	567.3 (38.2)	14.5 (15.6)	36.96 (19.8)	19.5 (13.1)	0.74 (17.2)	113 (13.5)	2.722
CV 159	564.9 (37.0)	14.5 (20.7)	34.55 (12.6)	20 (15.1)	0.72 (18.7)	104 (16.7)	2.561
BR 13 A X CV98/21	554.5 (56.7)	18.8 (20.4)	30.84 (26.9)	19.5 (19.3)	0.96 (29.8)	107 (17.7)	2.397
CV 33 S1	541.5 (54.7)	13.7 (24.4)	32.25 (21.9)	18.9 (29.6)	0.72 (18.3)	112 (22.3)	2.452
CV 98/2 X CV 136 EG	503.9 (35.4)	16.8 (28.4)	32.36 (18.1)	16.5 (17.9)	1.02 (34.4)	100 (27.4)	2.039
CV 175 White Flash	467.09 (41.1)	7.46 (20.9)	29.97 (13.3)	14.6 (15.7)	0.51 (11.1)	101 (15.6)	1.777
CV 174 Graffiti	461.8 (47.1)	10.8 (16.1)	32.98 (13.9)	17.5 (16.4)	0.62 (17.4)	110 (19.3)	2.198
CV 75 S3AC	453.5 (49.7)	11 (17.2)	35.54 (17.9)	18.1 (27.3)	0.61(22.5)	117 (15.8)	2.404
CV 24 S4	443 (55.9)	12.7 (23.0)	36.41 (24.2)	16.7 (23.8)	0.76 (32.4)	91 (26.5)	1.977
CV 98/2 X BR 13 S3	438.8 (84.4)	17.6 (24.4)	28.81 (28.1)	16.8 (29.4)	1.05 (34.2)	93 (17.7)	1.723
BR 41	378.3 (46.2)	10.2 (21.0)	36.8 (16.9)	17.2 (19.5)	0.59 (17.8)	113 (17.5)	2.184
BR 165 Marathon	319.8 (40.9)	14.1 (26.8)	3.52 (19.5)	12.28 (26.7)	1.2 (44.4)	76 (29.8)	0.061
CV 136 EG X CV98/2	317.4 (42.0)	17.2 (22.2)	29.22 (28.1)	14.8 (16.0)	1.17 (33.1)	98 (21.6)	1.410
CV 72 S2	305.7 (68.2)	8.7 (20.7)	31.7 (18.1)	15.4 (22.8)	0.56 (19.7)	92 (25.2)	1.470
BR 46	279 (39.0)	16.6 (18.1)	3.84 (17.3)	11.1 (23.7)	1.5 (28.2)	57 (21.5)	−0.342
BR 45 S1	266.9 (33.4)	22.2 (30.9)	3.18 (13.2)	8.47 (32.7)	2.7 (37.4)	58 (19.4)	−0.595
BR 17 S2	263.6 (56.1)	11.2 (28.3)	34.23 (18.8)	14.4 (22.0)	0.78 (21.2)	91 (23.4)	1.379
BR 129	226.4 (39.6)	18.2 (12.9)	3.13 (26.8)	7.89 (29.4)	2.3 (30.5)	49 (27.8)	−0.821
BR 128	217.7 (58.3)	18.2 (18.2)	2.93 (29.8)	9.49 (31.6)	1.9 (29.4)	54 (26.3)	−0.659
BR 122 Packman	212.8 (36.3)	12.8 (12.2)	3.14 (15.0)	7.78 (23.1)	1.9 (16.5)	46 (24.1)	−0.877
BR 126	188.3 (51.8)	16.6 (23.4)	2.87 (24.3)	7.7 (28.3)	2.2 (24.2)	46 (24.1)	−0.951
CV 19 S2 A	186.6 (41.3)	8.4 (17.5)	28.6 (16.7)	13.6 (15.1)	0.61 (18.2)	85 (24.8)	0.905
BR 106	164 (49.0)	16.5 (17.9)	3.34 (32.4)	8.25 (29.5)	2 (52.3)	46 (32.8)	−0.940
BR 94 S1	143.9 (42.2)	16 (29.0)	2.69 (22.7)	7.82 (29.0)	2.1 (22.6)	48 (26.7)	−1.008
BR 115 S1	109.5 (30.8)	15.5 (9.5)	2.64 (20.2)	7.88 (25.8)	2 (23.4)	41 (34.2)	−1.158
BR 36	63.1 (41.7)	16.9 (23.5)	2.76 (18.9)	4.74 (22.3)	3.6 (15.5)	27 (15.2)	−1.664
Brassica macrocarpa 5	36.7 (21.1)	8.2 (12.1)	14.5 (16.3)	3.4 (23.1)	0.23 (27.9)	12 (10.2)	−1.572
Brassica rupestris	33.3 (28.3)	27.6 (15.5)	16.2 (20.2)	3.1 (17.9)	0.19 (21.2)	14 (11.7)	−1.574
Brassica macrocarpa 3	31.2 (19.8)	18.6 (21.2)	10.8 (23.6)	2.4 (16.2)	0.22 (19.8)	15 (12.6)	−1.781
Brassica macrocarpa 1	30.9 (23.2)	15.4 (18.4)	7.3 (20.7)	3.1 (19.2)	0.42 (38.4)	9 (7.9)	−1.915
Brassica incana 1	30.3 (21.9)	21.1 (19.2)	25.7 (26.3)	3.8 (21.7)	0.15 (26.5)	12 (11.7)	−1.201
Brassica rupestris 3	29.8 (19.8)	20.5 (12.2)	16.9 (20.5)	4.2 (22.2)	0.25 (21.6)	13 (7.3)	−1.463
Brassica rupestris 2	27.5 (17.5)	18.4 (9.1)	21.6 (23.4)	3.9 (25.4)	0.18 (17.8)	17 (10.2)	−1.271
Brassica macrocarpa 8	27.2 (18.4)	13.2 (21.2)	8.5 (19.5)	2.9 (19.1)	0.34 (18.9)	14 (11.9)	−1.827
Brassica incana 3	25.1 (21.8)	19.6 (24.6)	19.3 (31.3)	2.7 (17.1)	0.14 (27.1)	15 (9.8)	−1.477
Brassica macrocarpa 7	24 (21.2)	21.5 (27.2)	11.4 (21.2)	2.5 (19.5)	0.22 (21.0)	10 (8.1)	−1.837
Brassica rupestris 6	22.7 (20.1)	26.3 (20.4)	20.7 (28.2)	2.1 (25.3)	0.1 (19.2)	9 (7.2)	−1.573
Brassica rupestris 7	22.1 (18.9)	20.6 (26.1)	18.5 (18.4)	2.5 (19.2)	0.14 (18.8)	10 (8.3)	−1.591
Brassica macrocarpa 2	21.7 (18.4)	15.8 (21.2)	8.2 (19.2)	3 (18.8)	0.37 (18.9)	12 (7.7)	−1.873
Brassica rupestris 4	21.6 (16.2)	19.8 (9.1)	7.3 (16.3)	2.1 (16.9)	0.29 (15.9)	11 (8.2)	−1.998
Brassica macrocarpa 6	21.6 (20.3)	17.6 (13.6)	21.4 (19.7)	2.1 (17.4)	0.1 (16.5)	15 (9.0)	−1.451
Brassica incana 2	21.5 (15.2)	20.3 (21.1)	19.6 (19.1)	3.1 (21.1)	0.16 (19.8)	12 (9.2)	−1.482
Brassica rupestris 5	20.5 (19.0)	20.8 (16.9)	16.3 (20.1)	2.2 (22.2)	0.13 (20.5)	12 (6.3)	−1.670
Brassica villosa 1	20.1 (18.2)	15.1 (12.1)	19.8 (19.2)	2.6 (18.4)	0.13 (19.3)	11 (9.2)	−1.514
Brassica rupestris 1	19.8 (16.1)	21.6 (19.5)	8.9 (16.2)	2.1 (14.2)	0.24 (16.0)	7 (6.1)	−2.001
Brassica macrocarpa 4	19.8 (17.2)	23.2 (20.3)	19.6 (23.3)	2.6 (21.8)	0.13 (23.2)	11 (8.9)	−1.545
Brassica incana 4	19.7 (9.1)	21.2 (23.2)	17.3 (21.2)	2.5 (18.8)	0.14 (20.2)	11 (8.0)	−1.627
Brassica villosa 2	19.2 (18.4)	14.5 (9.1)	18.1 (15.2)	2.2 (19.2)	0.12 (19.1)	10 (6.8)	−1.616

Legend: number in brackets indicates standard deviation.

Table 4. Correlation coefficients of single descriptors with the three main principal components (PCs).

	PC1	PC2	PC3
CW	0.508	0.06	0.229
CH	−0.032	0.998	0.010
CD1	0.438	0.022	−0.898
CD2	0.527	−0.022	0.251
CA	0.521	0.004	0.279
% variance	46.75	25.21	16.34

Morphometric traits were subsequently elaborated and correlated to the genetic data by statistical analysis.

Each SSR locus exhibited a different number of alleles among the accessions studied: BoTHL1 showed eight alleles, PBCGSSRBo39 eleven alleles, BoPLD1 six alleles, BoAP1 showed twelve alleles, and BoABI1 nine alleles. Allelic data were processed to measure genetic diversity for each locus within the different *Brassica* accessions examined and to calculate the H index (Table 5).

Table 5. Multiple regression on several loci heterozygosity indices of four plant growth parameters: CW—Curd weight, CD1—Curd inflorescence diameter; CD2—Curd Stem thickness and their First Principal Component (PC1).

	Estimate	Std. Error	*p*-Value
CW on H indices			
BoTHL1	95.48	128.87	0.4643
PBCGSSRBo39	170.24	130.62	0.2021
BoPLD1	−248.18	137.86	0.0888
BoAP1	142.67	99.97	0.1635
BoABI1	−178.45	117.18	0.1379
CD1 on H indices			
BoTHL1	4.5004	3.0624	0.1518
PBCGSSRBo39	−0.6356	3.1040	0.8391
BoPLD1	−6.9635	3.2759	0.0416
BoAP1	2.7333	2.3756	0.2587
BoABI1	−3.3929	2.7845	0.2322
CD2 on H indices			
BoTHL1	8.413	7.049	0.2417
PBCGSSRBo39	1.682	7.145	0.8154
BoPLD1	−19.056	7.541	0.0168
BoAP1	1.124	5.468	0.8386
BoABI1	−12.926	6.410	0.0525
PC1 on H indices			
BoTHL1	1.1348	0.8870	0.2102
PBCGSSRBo39	0.3260	0.8990	0.7193
BoPLD1	−2.2453	0.9488	0.0244
BoAP1	0.6661	0.6881	0.3405
BoABI1	−1.2391	0.065	0.1346

The correlation between CW and the five locus H index did not show significant *p*-value, although the BoPLD1 one was weakly significant (smaller than 0.10). On the other hand, significant correlations were observed among BoPLD1 H index and CD1, CD2, and PC1 (Table 5). The negative sign of the estimate coefficient confirms the association between the heterozygosity index and BoPLD1; when the H index increases the size of the inflorescence and the thickness of the stem decrease. The analysis also showed no significant correlation between the H index of the other loci (BoAP1, BoTHL1, BoAB1, PBCGSSRBo39) and inflorescence characteristics.

5. Discussion

The Di3A core collection describes the evolution of the domestication process from the *Brassica* wild species (n = 9) to the broccoli and cauliflower crops by comparing the main morphometric traits of the inflorescence and the allele diversity of the molecular primers utilized during human selection.

The domestication process is explained by the use of five SSR primers which show a wide range of alleles among the growing and wild species belonging to the *B. oleracea* complex species (n = 9). Some alleles, useful for increasing the inflorescence size, were unconsciously subject of selection by the growers in order to fix the hypertrophic inflorescence of broccoli and cauliflower. In addition, the broccoli and cauliflower domestication processes have been affected by the genetic flux among the *Brassica* wild relatives (n = 9), and the first domesticated sprouting broccoli was permitted to enlarge the inflorescence size gradually and define its shape of the hybrids F1 of broccoli and cauliflower [17]. In Branca et al. Fst was calculated in order to measure the genetic distance among accessions; the genetic diversity shown by the five SSRs primers utilized in relation to the *B. oleracea* complex species (n = 9) accessions, permitted us to classify them in relation to their domestication process (CWRs, landraces of cauliflowers and broccoli and their hybrids F1. MADS–box genes family includes several transcriptional factors involved in the growth and development of the inflorescence after its reproductive induction, flowering time, fruit development, and ripening [18]. During the last decades, several genomic studies were reported to explain the role played by some homeotic genes involved in the development of the hypertropic inflorescence, called head for broccoli and curd for cauliflower. Several genes such as *apetala* 1 (AP1) were reported to be involved in the inflorescence structure controlling its meristematic development. The transcript of AP1 gene (RefSeq ID: XP_013590290.1) was described by Sheng et al., 2019 as showing high levels of expression in different tissues and in particular in the curd and the flower [19]. These genes are related to the development of the reproductive organs and they belong to the MADH-box genes family [20]. *BoCal* is one of the related genes involved in curd formation; the mutant alleles seem to stop flower development and a simple genetic model has been proposed [9].

BoPLD1 marker is located in the fifth chromosome of *B. oleracea* from 46,037,340 bp to 46,037,606 bp in an untranscribed region (accession: LR031877), near the region encoding Phospolipase D (UniProtKB-A0A3P6FGA7). This catalytic enzyme is encoded from BOLC5T33808H gene and is involved in glycerolphospholipids hydrolysis at the terminal phosphodiesteric bond.

Taking into consideration the stem diameter (CD2) and the diameter of the inflorescence (CD1), the correlation between their values and the H index is significant only for the BoPLD1 locus while it is weakly significant with respect to the weight of the inflorescence (Table 5). The analysis of the negative correlation coefficient between these two inflorescence morphometric traits (CD1 and CD2) permits us to deduce that the more the H index associated with BoPLD1 increases, and the related locus tends to show higher heterozygosity, the more such a parameter affects the inflorescence size. This could be correlated with the observation that in the CWRs there is greater heterozygosity than in the hybrids F1 but the size of the inflorescence, its stem, and its weight decrease for the former. The sequence hosting the microsatellite placed in the initial portion of the gene *Bo5g126670,* just before the first exon, could not exclude the presence of a repeat affecting the transcription of the gene itself.

The data acquired consent to delineate the next steps of this study sequencing the polymorphisms present in the upstream region of the *Bo5g126670* gene that could be involved in the inflorescence hypertrophy of the *B. oleracea complex species* (n = 9). These variations could be used for marker-assisted selection (MAS) and for individuating in advance, during the breeding program utilized CWRs—the individuals who express hypertrophic inflorescence are an object of interest for further field evaluation.

6. Conclusions

BoPLD1 marker heterozygosity (H index) shows significant interaction with several inflorescence morpho-biometric characteristics and when BoPLD1 alleles tend to homozygosity an increase of inflorescence and curd size are observed. These results permit us to continue to investigate by sequencing these primers to individuate the SNPs useful for distinguishing the broccoli types with hypertrophic inflorescence during the organic breeding programs. The crossing plans among the broccoli breeding lines and the *Brassica* wild relatives, aiming to transfer forgotten alleles during the domestication process, will be useful for increasing the resistance against biotic and abiotic stresses, and for nutritional, organoleptic and nutraceutical traits. The molecular marker will reduce the cost of evaluation field transplanting with only the selected individuals expressing the broccoli inflorescence phenotype, reducing the number of individuals to grow and to analyze.

Author Contributions: Conceptualization, S.T. and F.B.; methodology, F.B., C.D.G., M.G.; formal analysis, F.D.S.; investigation, S.T.; resources, F.B.; data curation, S.T.; writing—original draft preparation, F.B., C.D.G., S.T.; writing—review and editing, F.B., M.G.; funding acquisition, F.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the project BRESOV (Breeding for Resilient, Efficient and Sustainable Organic Vegetable production) funded by EU H2020 Programme SFS-07-2017. Grant Agreement n. 774244.

Conflicts of Interest: The authors declare no conflict of interest.

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