A MULTIVARIATE APPROACH TO EXPLORE THE GENETIC VARIABILITY IN THE F$_2$ SEGREGATING POPULATION OF A TOMATO SECOND CYCLE HYBRID

VARIABILIDAD EN LA POBLACIÓN F$_2$ SEGREGANTE DE UN HÍBRIDO DE TOMATE DE SEGUNDO CICLO

Cabodevila V.G.[1,2,3,*], Picardi L.A.[1,3,4], Pratta G.R.[1,2,3]

[1] Cátedra de Genética, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Campo Experimental Villarino, CC14 S2125ZAA, Santa Fe, Argentina.
[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
[3] Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR).
[4] Consejo de Investigaciones de la Universidad Nacional de Rosario (CIUNR).

* corresponding author: victoria.cabodevila@unr.edu.ar

ABSTRACT

Segregating progeny from the tomato Second Cycle Hybrids (SCH) that were obtained from crossing RIL (Recombinant Inbred Lines) allows the detection of new genetic combinations that could increase genetic variability in F$_2$ populations. The objectives of the present study were to evaluate eleven tomato quality traits in a segregating F$_2$ population obtained from a SCH and, then, to characterize the molecular diversity by six AFLP (Amplified Fragment Length Polymorphism) primer combinations. Different multivariate analyses were used to assess the degree of concordance among these two approaches to detect genetic variability. Sixty-nine F$_2$ plants were obtained by selfing the SCH (ToUNR18xToUNR1). The parental RIL were derived from an interspecific cross between *S. lycopersicum* cv. Caimanta and the accession LA722 from *S. pimpinellifolium* after five cycles of antagonist and divergent selection for fruit weight and fruit shelf life. Principal Components Analysis (PCA) was applied to these data and we found that the first two components explained 77 % of variability. The molecular characterization showed 62 % of polymorphic bands. The Principal Coordinate Analysis (PCoA) showed that the first ten coordinates explained 75 % of variability. The Generalized Procrustes Analysis (GPA) showed a consensus between morphological and molecular data of 65 %. High values of broad sense heritability (H^2) were found for all traits together with a high level of molecular polymorphism. The morphological and molecular data showed a high consensus proportion suggesting that it could be possible to detect QTL for these fruit traits exploring this new population.

Key words: plant breeding, plant genetic resources, fruit quality, AFLP, multivariate analysis

RESUMEN

Las generaciones segregantes de Híbridos de Segundo Ciclo (HSC) de tomate, obtenidos por cruzamiento entre RIL (Líneas Endocriadas Recombinantes), constituyen otro acervo genético que permitiría detectar nuevas combinaciones genéticas. Once caracteres cuantitativos de los frutos se evaluaron en una población F$_2$ obtenida a partir de un HSC y luego se caracterizó la variabilidad molecular mediante seis combinaciones de iniciadores de AFLP (Polimorfismo para Longitud de Fragmentos Amplificados). Se utilizaron sesenta y nueve individuos F$_2$ obtenidos por autofecundación del HSC (ToUNR18xToUNR1). Las RIL parentales fueron obtenidas a partir de un cruzamiento interespecífico entre *S. lycopersicum* cv. Caimanta y la introducción LA722 de *S. pimpinellifolium* luego de cinco ciclos de selección divergente y antagonista para peso y vida poscosecha de los frutos. El Análisis de Componentes Principales mostró que los primeros dos componentes explicaban 77 % de la variabilidad, la caracterización molecular reveló 62 % de bandas polimórficas, y el Análisis de Coordenadas Principales evidenció que las primeras diez coordenadas explicaban 75 % de la variabilidad. El Análisis de Procrustes Generalizado mostró un consenso entre la información fenotípica y molecular de 65 %. Para la mayoría de los caracteres se encontraron valores altos de heredabilidad en sentido amplio junto con el alto nivel de polimorfismo molecular. Este alto consenso entre la información fenotípica y molecular sugiere que sería posible la detección de QTL para estos caracteres relacionados a calidad de fruto.

Palabras clave: mejoramiento vegetal, recursos genéticos vegetales, calidad de fruto, AFLP, análisis multivariado

Article 1 - research

INTRODUCTION

The cultivated tomato (*Solanum lycopersicum* L.) is an economically important crop over the world but the migratory process during domestication together with crop improvement have reduced the genetic variability of cultivated germplasm (Blanca *et al.*, 2015). Nevertheless, some increase of genetic variation is present due to the introgression of genes for biotic stress resistance from wild species (Sim *et al.*, 2012). On the other hand several experiments have showed that wild genotypes have also differential fruit quality traits. Zorzoli *et al.* (1998) showed that fruits from wild tomato species (*S. pimpinellifolium*) had longer shelf life than commercial genotypes. Pratta *et al.* (1996) have evaluated different tomato wild and cultivated accessions for fruit shelf life and they have found that the accession LA722 (Tomato Genetic Resources Centre, Davis, USA) was an appropriate genotype to be crossed with an Argentinean cultivar of *S. lycopersicum* (cv. Caimanta) to improve fruit shelf life. From this approach seventeen RIL (Recombinant Inbred Lines) were obtained (Rodríguez *et al.*, 2006). For the present experiment, a Second Cycle Hybrid (SCH) was obtained by crossing some of the selected RIL (Kearsey and Pooni, 1996). As De Toledo *et al.* (1984) have pointed out, these SCH could be an interesting genetic material, with novel genotypes which could have a better performance than the best of the recombinant inbred lines or even the original F_1. In the segregating progeny of the SCH, new genetic combinations can be found, which can be identified by DNA markers. In this way, molecular and morphological information could be a previous step to QTL detection. In another experiment in tomato, Marchionni Basté *et al.* (2010) found positive General Combining Aptitude values in a diallel analysis for three RIL, including ToUNR18 and ToUNR1. These results have suggested that these RIL could be potential parents to improve important tomato fruit traits such as weight, shelf life, colour, soluble solids content and firmness. The objectives of the present study were to evaluate eleven phenotipic fruit traits in the segregating F_2 population from the SCH (ToUNR18xToUNR1) and, then, to characterize the molecular diversity in this generation by AFLP patterns. We consider that multivariate analyses applied on this information could be the first step to assess putative QTL for the evaluated traits in this genetic material.

MATERIAL AND METHODS

Plant material

The assay was carried out at the experimental field station "J.F. Villarino", Universidad Nacional de Rosario, Argentina (33° S, 61° W). Sixty-nine F_2 plants were obtained by selfing the SCH (ToUNR18xToUNR1). After five cycles of antagonist and divergent selection for weight and fruit shelf life (Rodriguez *et al.*, 2006), parental RIL, ToUNR1 and ToUNR18 were derived from an interspecific cross between *S. lycopersicum* cv. Caimanta (C, a commercial Argentinean genotype) and *S. pimpinellifolium* LA722 (P, a wild genotype accession, TGRC, Davis, USA). Both RIL and their SCH (the uniform second cycle genotypes, USCG) together with C, P and their interspecific F_1 CxP (the uniform first cycle genotypes, UFCG) were included in the assay as testers.

Phenotypic traits

Fruit traits were evaluated at two different stages: a) breaker (first visible carotenoid accumulation (Giovannoni, 2004)): Weight (W, in g), Height (H, in cm), Diameter (D, in cm), Shape index (S) as the ratio between H and D and fruit Shelf Life (SL, in days) as the number of days from harvest to fruit softening stored at 25 ± 3 °C ; b) ripening (approximately 90 % red surface): Firmness (F) measured on two opposite equatorial sides with a digital firmness tester (Durofel, DFT 100), pH, from the fruit juice, Soluble Solids (SS, in Brix degrees) as the percentage of fructose and glucose, titratable acidity (TA, in g) as the g of citric acid in 100 g of the homogenized juice, the Reflectance Percentage (L) and the Absorbance Index (a/b) as the ratio between the absorbance at 540 nm (a) and the absorbance at 675 nm (b). The L, a, and b traits were measured with a chromameter (Konica–Minolta).

Molecular data

Young leaves were collected from the F_2 generation and all tester plants. DNA was extracted using a commercial kit (Wizard ® Genomic DNA Purification Kit). The DNA quality and concentration was checked on 1 % agarose gel and compared with the λ phage. The AFLP technique was applied following a standard protocol (Blears *et al.*, 1998) with minor changes. The genomic DNA was digested using two restriction enzymes: *EcoR*I

and *Mse*I. For ligation, double-stranded nucleotide adapters complementary to the restriction sites (*Mse*I and *Eco*RI Adapter) were used. The pre-amplification was made using *Mse*I+1 (5'-GATGAGTCCTGAGTAAC-3') and *Eco*RI+1 (5'-GACTGCGTACCAATTCA-3'). Six AFLP primers combinations were used for the selective amplification. These combinations were selected because they produced high number of bands and a large percentage of polymorphism in the testers (Cabodevila *et al.*, 2013). The combinations were:
F: 5'-GATGAGTCCTGAGTAACGT-3'/
5'-GACTGCGTACCAATTCACT-3';
J: 5'-GATGAGTCCTGAGTAACTA-3'/5'GACTGCGTACCAATTCAGA-3';
H:5'-GATGAGTCCTGAGTAACAG-3'/5'GACTGCGTACCAATTCACT-3';
HH:5'-GATGAGTCCTGAGTAACTA-3'/5GACTGCGTACCAATTCATT-3';II:
5'-GATGAGTCCTGAGTAACTG-3'/5'-GACTGCGTACCAATTCATT-3' and JJ:
5'-GATGAGTCCTGAGTAACGT-3'/5'-GACTGCGTACCAATTCATT-3'. A PCR touchdown was used. Separation of the amplification fragments was done in 6 % polyacrylamide gels and the visualization was made using silver staining (NO_2Ag).

Data analysis
The normality of phenotypic traits distribution was verified by the Shapiro-Wilk's test. Parental means were first compared between them and, then, each of them was compared with their hybrid using the Duncan's test. The dominance degree (d/a) was calculated in UFCG and in USCG for those traits that showed significant differences between parents. Also phenotypic distribution was explored for all quantitative traits. Broad sense heritability (H^2) was estimated according to Mather and Jinks (1971), to assess the degree of genotypic component in this new phenotypic variance. A Principal Component Analysis (PCA) was performed for the F_2 individuals including all morphological fruit traits. Following Pratta *et al.* (2000), data were not standardized. Molecular characterization included the total number of bands, the polymorphic bands and the *de novo* bands; following, the degree of polymorphism (in percentage) was calculated. We considered that F_1 *de novo* bands were those which were either present (1) or absent (0) in both RIL but either

absent or present, respectively, in the SCH. Bands either absent or present in both RIL and in the SCH, but with 10 % or more of either presence or absence in the F_2, were considered F_2 *de novo* bands. The χ^2 test was used to verify the Mendelian segregation of the polymorphic bands. Bands were tested under the 3:1 segregation hypothesis (3 present: 1 absent or 3 absent: 1 present, for presence in one of the RIL and in the F_1, or absence in one of the RIL and also in the F_1). The *de novo* bands in F_1 were contrasted to a 1:1 segregation. The *de novo* bands in F_2, were contrasted to a 3:1 segregation (3 present: 1 absent or 3 absent: 1 present for more presence than absence or more absence than presence in F_2). A Principal Coordinate Analysis (PCoA) was carried out using all the bands that had a Mendelian segregation and with the Jaccard (1-S) distance (Jaccard, 1901). A Generalized Procrustes Analysis (GPA) was performed using the Gower distance (Gower, 1966; 1975), in order to measure the consensus between the morphological and molecular information. The PCA, PCoA and GPA were performed using InfoStat (Di Rienzo *et al.*, 2016).

RESULTS

Significant differences between C and P genotypes were found for all fruit traits, excepting SS and pH traits. Also in the UFCG group significant differences were found for L, TA, D, H, W and F traits between these parents and their interspecific CxP F_1 progeny (see Figure 1 Sections A and B). As it can be seen in Table 1, complete dominance was observed for a/b, S and SL traits, whereas partial dominance was observed for TA, D, H, W and F. Only for the L trait a negative overdominance was detected.

Regarding to the USCG, significant differences between the parental RIL (ToUNR18 and ToUNR1) were observed for L, SS, TA, D, H and S traits. Only for H trait a significant difference was observed between these parental RIL and their F_1 (ToUNR18xToUNR1) (Figure 1 Sections A and B). Hence, the dominance degree in the USCG indicated a complete dominance for L, SS, TA, D, and S. A negative overdominance was found for H.

Transgressive segregation was observed for all traits in the F_2 progeny as it can be seen in Figure 1 Sections A and B. As Singh *et al.* (2013) have pointed out, transgressive segregation is an indicator of the broadening of the genetic variance.

The C and P genotypes, which had the highest and lowest values for the W, D and H traits, were never surpassed by any F_2 individual. For the SL, F and S traits, we found F_2 genotypes that surpassed the average values of genotype C; for the TA trait, some genotypes had values below P; for the L trait, some genotypes had values above CxP and, finally, for the a/b trait, some genotypes had values below P and CxP.

All traits were normally distributed in the F_2, except for the TA trait. This trait was transformed for this analysis by the Log_{10}. Broad sense heritability (H^2) values for all traits are shown in Table 1. The TA, SS and pH traits had the higher values (0.98, 0.94 and 0.90, respectively).

The segregating F_2 generation analysed by PCA for all fruit traits showed that the first two components accounted for 77 % of the variation (PC1= 41 %, PC2= 36 %). The PC1 was mainly explained by the F trait and the PC2 by the SL and W traits (Figure 2a). The cophenetic correlation coefficient was 0.93. The molecular characterization showed 68 out of a total of 110 bands were polymorphic (62 %). A section of a polyacrylamide gel is shown in Figure 3. There were 51 *de novo* bands only in the second cycle genotypes: 25 of them were *de novo* in the F_1 (bands that were present in the RIL and absent in the F_1, or absent in the RIL and present in the F_1) and the other 26 bands were *de novo* in the F_2 (either present or absent in the RIL and F_1, and with 10 % or more of absence or presence in the F_2). Twenty-nine of the 68 polymorphic bands adjusted to the expected Mendelian segregation (43 %). The PCoA using the Jaccard distance (1–S) for the six AFLP primer combinations and the bands with the expected segregation showed a large variation (Figure 2b); as a consequence, ten coordinates were necessary to explain75 % of the variability (see the wide distribution of genotypes in Figure 2b).

The PCA and the PCoA analysis allowed to carry out the joint analysis (GPA). The first four components explained 77 % of the variability (CP1= 35 %, CP2= 22 %, CP= 13 %, CP4= 6 %), with a consensus of 65 % (Figure 4).

Table 1. Environmental (σ2E), genotypic (σ2G) and phenotypic variances (σ2P), broad sense heritability (H2) and dominance degree (d/a) for quantitative fruit traits. D: Diameter; H: Height; W: Weight; S: Shape; SL: Shelf Life; F: Firmness; a/b: Absorbance Index; L: Reflectance Percentage; SS: Soluble Solids; TA: Titratable Acidity.

	σ^2E	σ^2G	σ^2P	H^2	d/a	
					UFCG	USCG
D	0.2888	0.1022	0.3910	0.26	-0.63	-1.00 (L18)
H	0.2195	0.1490	0.3685	0.40	-0.38	-2.78
W	48.4080	10.6337	59.0417	0.18	-0.90	-
S	0.0175	0.0036	0.0211	0.17	1.00 (P)	-1.00 (L1)
SL	41.8047	31.7570	73.5617	0.43	1.00 (P)	-
F	47.5928	55.5638	103.1566	0.54	-0.09	-
a/b	0.0167	0.0187	0.0354	0.53	1.00 (P)	-
L	2.0233	3.1314	5.1547	0.61	-2.30	-1.00 (L1)
pH	0.0075	0.0657	0.0732	0.90	-	-
SS	0.1108	1.7087	1.8195	0.94	-	1.00 (L1)
TA	0.0004	0.0188	0.0192	0.98	-0.48	1.00 (L1)

UFCG: Uniform First Cycle Genotypes; USCG: Uniform Second Cycle Genotypes; (P): the F_1 does not differ from S. *pimpinellifolium* accession LA722; (L1): the F_1 does not differ from RIL ToUNR1; (L18): the F_1 does not differ from RIL ToUNR18; -: the parents do not differ between them.
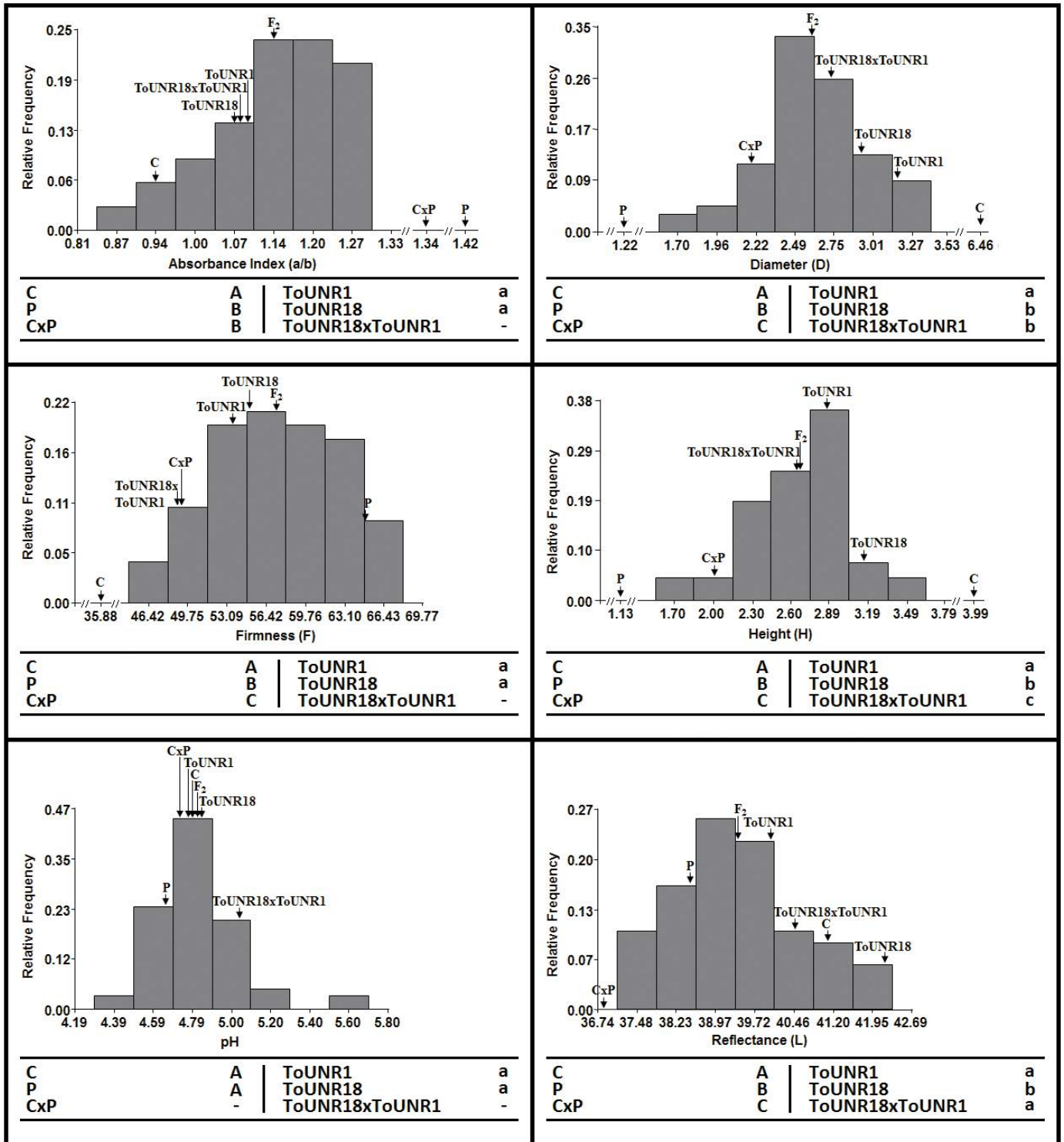
Figure 1 (Section A). Histograms for quantitative fruit traits in the F$_2$ population

The means for *Solanum lycopersicum* cv. Caimanta (C), *S. pimpinellifolium* accession LA722 (P), F1 CxP, ToUNR1, ToUNR18, SCH (ToUNR18xToUNR1) and F2 from this SCH are shown in the graphics. Different uppercase letters mean significant differences between UFCG by Duncan's test (p<0.05). Different lowercase letters mean significant differences between USCG by Duncan's test (p<0.05).
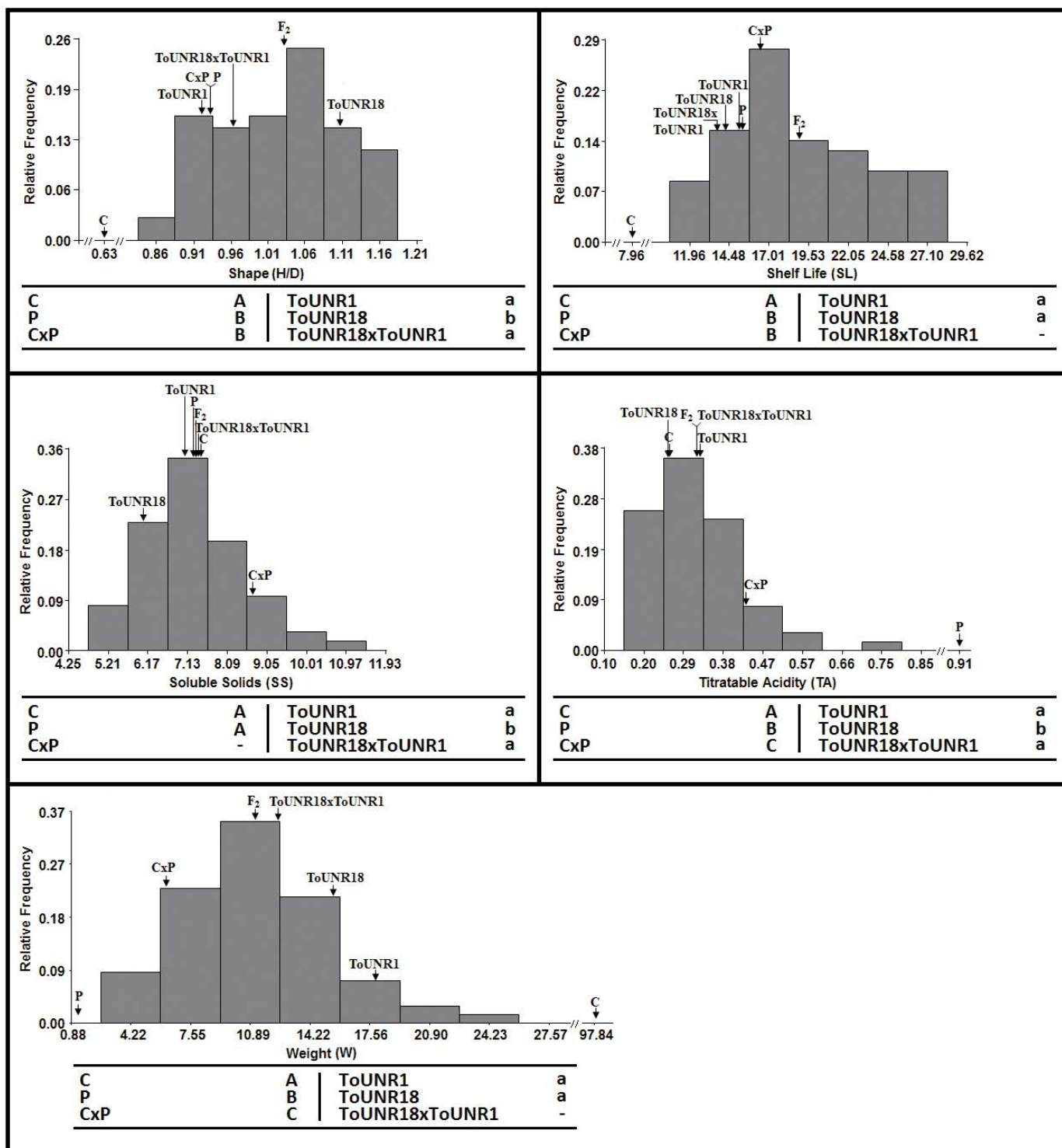
Figure 1 (Section B). Histograms for quantitative fruit traits in the F_2 population

The means for *Solanum lycopersicum* cv. Caimanta (C), *S. pimpinellifolium* accession LA722 (P), F_1 CxP, ToUNR1, ToUNR18, SCH (ToUNR18xToUNR1) and F_2 from this SCH are shown in the graphics. Different uppercase letters mean significant differences between UFCG by Duncan's test (p<0.05). Different lowercase letters mean significant differences between USCG by Duncan's test (p<0.05).
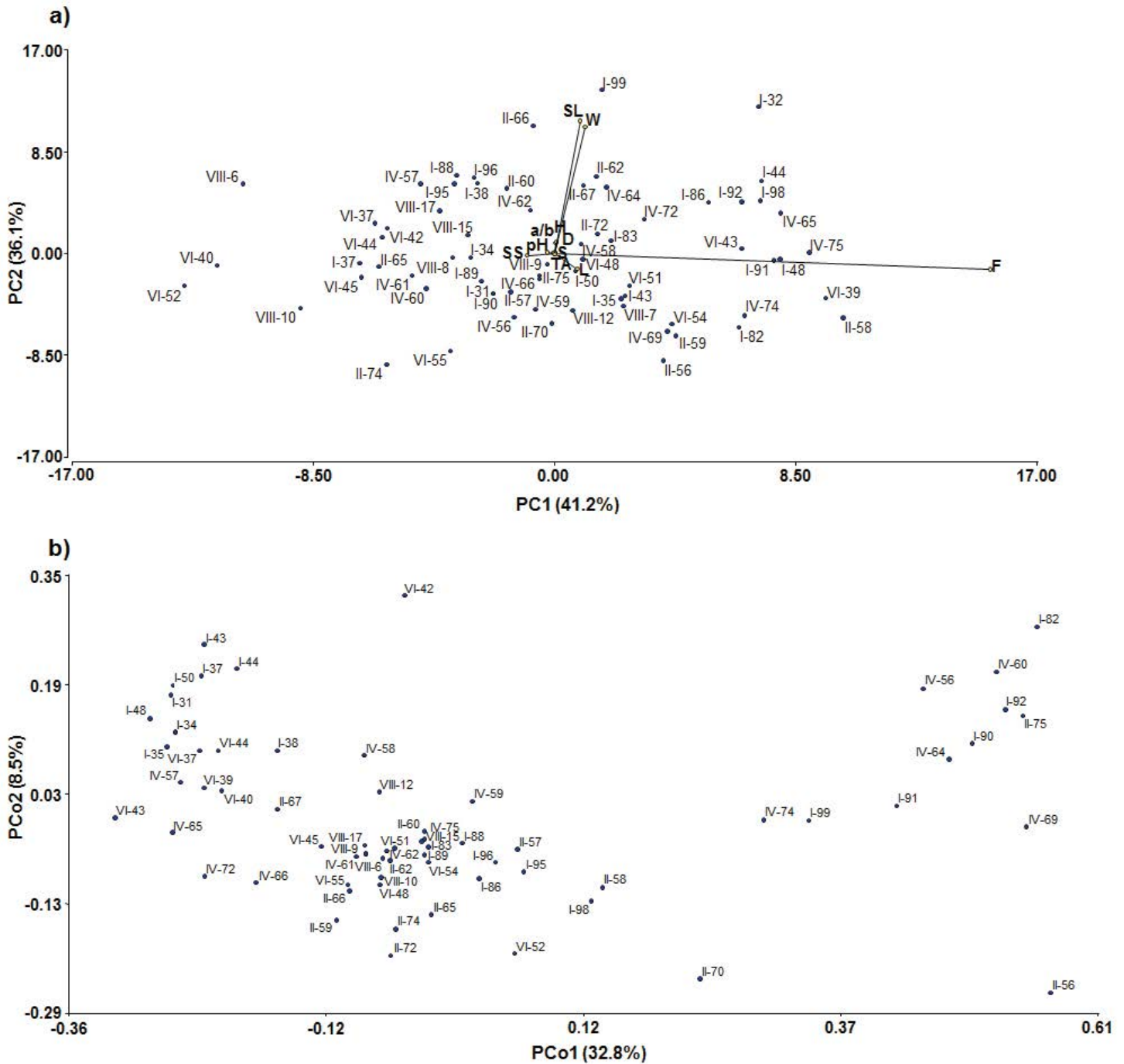
Figure 2. Biplot of: a) first two Principal Components (PC1 and PC2) of the Principal Component Analysis (PCA); b) first two Principal Coordinates (PCo1 and PCo2) of the Principal Coordinate Analysis (PCoA). Reflectance Percentage (L), Absorbance Index (a/b), Soluble Solids (SS), Height (H), Shape (S), Shelf Life (SL), Diameter (D), Weight (W), Titratable Acidity (TA) and Firmness (F).

In b) VIII-7 and VIII-8 genotypes are at the same point than VIII-9, and I-32 genotype is at the same point than? I-34.
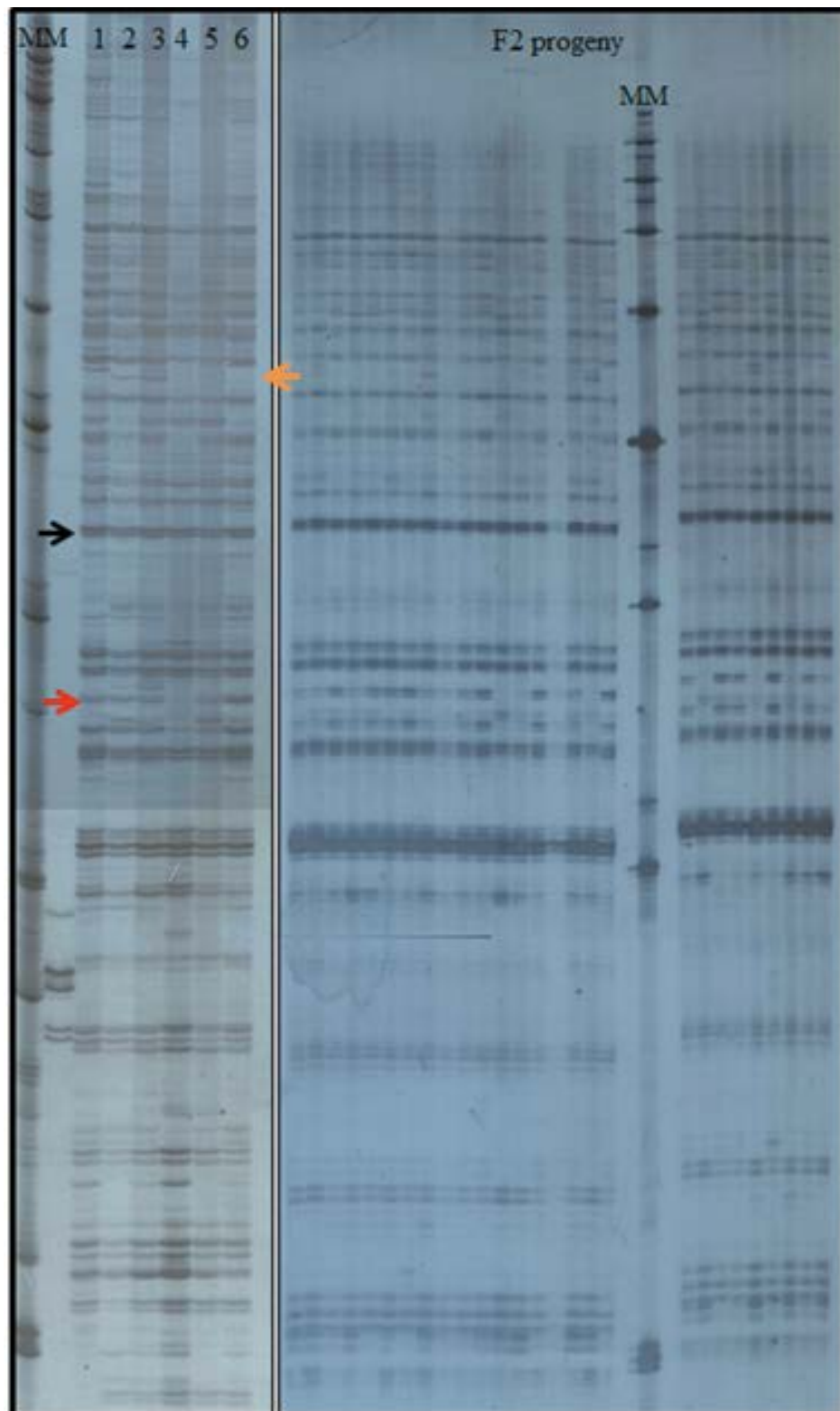
Figure 3. Two sections of a polyacrylamide gel of UFCG, USCG and some of the F_2 progeny for the J combination.

MM: Molecular marker; 1: *S. lycopersicum* cv. Caimanta (C); 2: *S. pimpinellifolium* accession LA722 (P); 3: CxP; 4: ToUNR1; 5: ToUNR18; 6: SCH: ToUNR18xToUNR1. Black arrow=monomorphic band (present in the UFCG, USCG and F_2 genotypes); red arrow= polymorphic band (present in UFCG, ToUNR18, SCH: ToUNR18xToUNR1 and in some F_2 genotypes); orange arrow= *de novo* band in F_2 (absent in USCG and present in some F_2 genotypes).
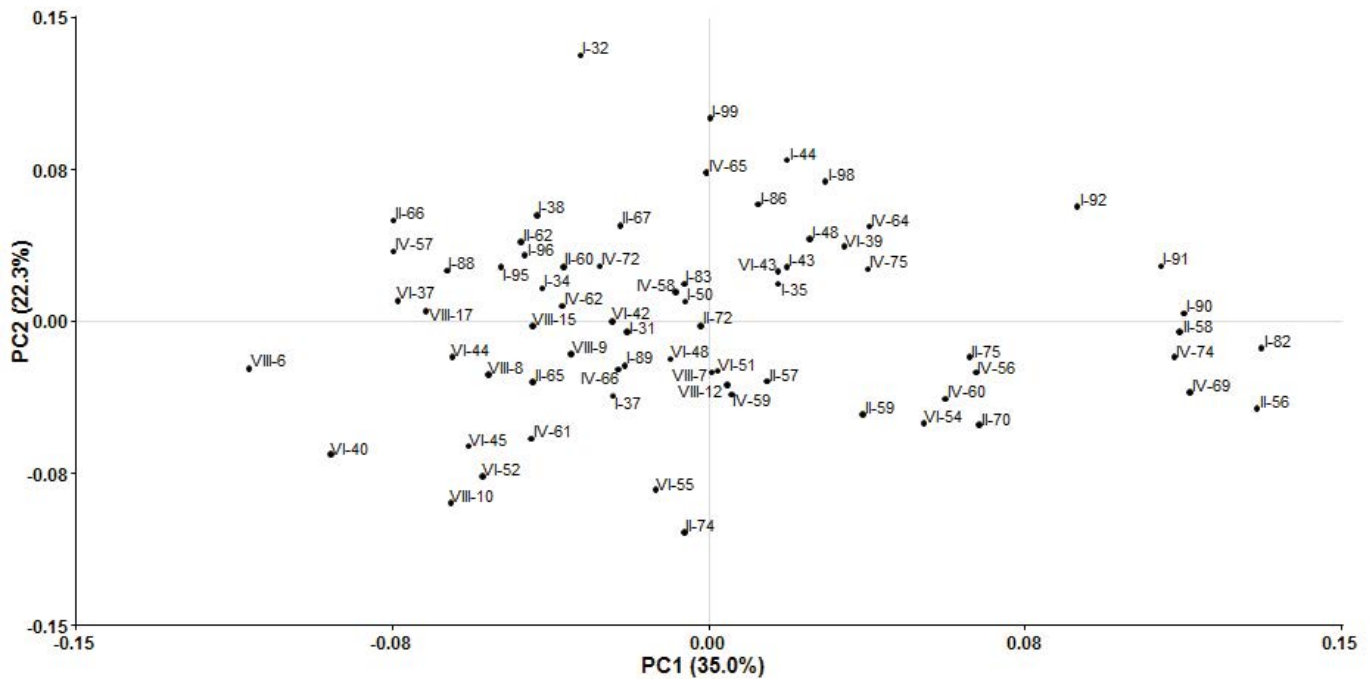
Figure 4. Consensus for the Generalized Procrustes Analysis (GPA) for eleven quantitative fruit traits and six AFLP primer combinations in the F_2 progeny .

DISCUSSION

In the present study, the F_2 population of a SCH among RIL, ToUNR18 and ToUNR1 was morphologically and molecularly characterized. The study of the association between morphological and molecular data is a first step to evaluate the level of agreement between the two types of data for eventual QTL detection. According to the results of the genetic analyses, complete and partial dominance were the more frequent types of gene action for these traits in the UFCG. Overdominance was only detected for the L trait. Similar results were observed in USCG, where complete dominance was observed in this group for L, SS, TA, D, and S, and overdominance for H. In both groups, UFCG and USCG, aditivity gene action seems to be absent and overdominance was scarce. Consequently in both, USCG and UFCG, the most frequent type of gene action was non–additive (including complete dominance, partial dominance and overdominance). Possibly, changes in type of gene action over generations for different traits could be due to the effect of selection, recombination and

chromosomal rearrangement, which could generate novel genotypic variation. Similar results were found by Liberatti *et al*. (2013) in another three SCH, where the most frequent type of gene action was non–additive either with complete dominance, partial dominance or overdominance.

Transgressive segregation was observed in the F_2 population for all traits. Broad sense heritability exhibited high values for all traits, with the highest values for pH, SS and TA, and the lowest values for S and W (the method utilized to estimate this parameter does not allowed to obtain the standard error and, in this way, to have more data about the reliability of these estimations).

Blears *et al*. (1998) indicated that one of the main advantage of AFLP markers is the number of *loci* detected per reaction. In this study, the six AFLP primer combinations revealed 110 bands, 68 of which were polymorphic (62 %). There were 51 de *novo* bands considering only the second cycle genotypes: 25 of them were *de novo* in the F_1 and the other 26 bands were *de novo* in the F_2; these bands could account for genetic changes occurring upon the crossing between cultivated and wild germplasm and their derived generations (Ercolano *et al*., 2004; Wang, *et al*., 2005; Vela *et al*., 2011; Paule *et al*., 2012).

The PCoA showed that ten coordinates were necessary to explain 75 % of variability and, finally, the combination of the morphological and molecular data, performed by PGA, showed 65 % of consensus. Mahuad *et al.* (2013) found 75 % of consensus between 11 quantitative traits and molecular data (SRAP) for 18 uniform genotypes (containing 10 SCH, five RIL and parentals of these RIL), suggesting that the GPA allowed the verification of the consensus between the variability of phenotypic traits and molecular markers. On the other hand, Bramardi *et al.* (2005) showed that General Procrustes Analysis was the most precise technique to cluster entries of cucumber (*Cucumis sativus* L.). In the tomato F_2 generation under study, the PCA demonstrated that the first two components explained 77 % of the observed variability. The correlation between molecular and morphological diversity indicate linkage disequilibrium between molecular markers and those genes that control morphological traits (Baranger *et al.*, 2004). The SCH may be unique genotypes which are capable of outperforming the best of the recombinant inbred lines or the original F_1. As we have shown, a high level of molecular polymorphism was detected in this experiment; moreover, the morphological and molecular data showed a high consensus proportion. Hence QTL detection in the SCH segregating generation through the application of multivariate techniques would be a good first approach for identifying chromosome regions controlling quantitative traits.

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