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Tomato Second Cycle Hybrids Differ from Parents at Three Levels of Genetic Variation

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ABSTRACT

Second Cycle Hybrids (SCHs) are produced by intercrossing Recombinant Inbred Lines (RILs) derived from the F_2 generation of two homozygous parents. Three tomato (*Solanum lycopersicum*) SCHs were obtained by crossing six RILs derived from an interspecific cross *S. lycopersicum* x *S. pimpinellifolium*. The objective was to characterize these SCHs and their parental RILs at different levels of genetic variation to understand the genetic causes underlying outperforming of these new genetic combinations. Fruit quality traits, pericarp polypeptide profiles at four ripening stages and AFLP were assessed. The degree of genetic determination and the degree of dominance were calculated for phenotypic traits, and the molecular polymorphism was evaluated for polypeptide and AFLP profiles. SCHs contributed with extreme values for some fruit quality traits, for which non additive gene actions were prevalent. Molecular differences were higher for polypeptide than for AFLP profiles, *de novo* bands being relevant to explain the increase in SCHs polymorphism. Cluster analysis showed different association among SCHs and RILs according to the level of genetic variation under study, providing a first advance for understanding the biological basis underlying the SCHs outperforming.

Keywords: amplified fragment length polymorphism, fruit quality, plant breeding, plant genetic resources, polypeptide profiles
Abbreviations: a/b, tomato fruit chroma index; **AFLP**, amplified fragment length polymorphism; **D**, tomato fruit diamenter; **d/a**, degree of dominance; **DGD**, degree of genetic determination; **F**, tomato fruit firmness; **H**, tomato fruit height; **L**, tomato fruit reflectance percentage; **RILs**, recombinant inbred lines; **S**, tomato fruit shape index, **SCHs**, second cycle hybrids; **SL**, tomato fruit shelf life; **SS**, tomato fruit soluble solids content; **TA**, tomato fruit titratable acidity

INTRODUCTION

Second Cycle Hybrids (SCHs) are produced by hybridizations among Recombinant Inbred Lines (RILs) derived from the F_2 generation of a cross between two homozygous parents (Hill *et al.* 2003). Due to some SCHs usually outperform the extreme RILs or the original F_1, they are especially valuable in plant breeding (Ipsilandis *et al.* 2006).

The cultivated tomato (*Solanum lycopersicum*) is a narrow genetic basis crop in which fruit quality is decisive for the acceptation of new varieties by the producers and farmers. Less than 5% of the available genetic variation exists in tomato cultivars and the remainder is found in wild species of the genus (Hu *et al.* 2012). Following this statement, 17 RILs from an interspecific cross between the Argentinean cultivar Caimanta of *S. lycopersicum* and the accession LA722 of *S. pimpinellifolium* were developed by Rodriguez *et al.* (2006a) with the aims of broadening the genetic variability for several fruit quality traits and obtaining new varieties to release in productive systems. RILs were obtained during six cycles of selfing and divergent-antagonistic selection for fruit weight and shelf life but also showed discrepant mean values for other plant and fruit traits (Rodriguez *et al.* 2006b). Hence this original germplasm became a promising source for continuing the exploration of favourable new genetic combinations involved in fruit quality through SCHs.

Phenotypic and molecular characterization is a powerful tool to manage and select genotypes in a breeding program, especially when wild germplasm was incorporated (Pereira da Costa *et al.* 2009). Currently breeders are able to assess the agronomic performance of the developed materials and to complement them with molecular studies. Polypeptides and DNA profiles are easily obtained with standard protocols and each of these analyses (field evaluations, polypeptide and DNA profiles) represents assessment at different levels of genetic variation: phenotypic attributes, gene expression, and genome structure and organization (Stevens 2008). In consequence, they should provide essential information for understanding the better performance of SCHs.

The objective of this work was to characterize at different levels of genetic variation three SCHs obtained by crossing six RILs developed from an interspecifc tomato cross (*S. lycopersicum* × *S. pimpinellifolium*), to understand the genetic causes underlying outperforming of these new genetic combinations.

MATERIALS AND METHODS

Plant material

Field assays were carried out at the experimental station "José F. Villarino" (Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Zavalla, Argentina, 33°S 61°W). Six RILs (TOUNR1, TOUNR5, TOUNR6, TOUNR9, TOUNR15, and TOUNR18) were chosen to produce the three SCHs (TOUNR1xTOUNR5), (TOUNR18xTOUNR6), and (TOUNR15xTOUNR9), according to the phenotypic and molecular characterization reported by Rodriguez *et al.* (2006b, field evaluation), Gallo *et al.* (2010, polypeptide profiling) and Pratta *et al.* (2011, DNA profiling). Fifteen seeds of each RILs and SCHs were germinated in seedling trays and the plants were then grown under greenhouse conditions in a complete randomised design. The total number of plants was 90 (10 per genotype) and the total number of harvested fruits was 1400.

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Original Research Paper

Assessment of fruit quality traits

Phenotypic characterization was made on breaker fruits (Giovannoni 2004) evaluated for height (H, in cm), diameter (D, in cm), shape index (S, ratio H/D), weight (W, in g), shelf life (SL, measured as the number of days elapsed from harvesting to the first symptoms of deterioration in fruits stored at 25±3°C, according to Schuelter *et al.* 2002). Also, red fruits (Giovannoni 2004) were evaluated for soluble solids content (SS, in ºBrix, determined with a hand refractometer in the homogenised juice from the pericarp tissue), pH, titratable acidity (TA, g of citric acid/100 mL of the homogenised juice from the pericarp tissue), color (measured by the reflectance percentage L and the chroma index, or a/b ratio, where a and b are the absorbencies at wave length of 540 and 675 nm, respectively, measured with a chromameter CR 300), and firmness (F, determined with a Fruit Pressure Tester -12.5 N- type Shore A with a tip of 0.10 in a 0–100 scale).

Assessment of total polypeptide and AFLP profiles

Total pericarp polypeptides at mature green (MG), breaker (B), red ripe on plant (RR) and red ripe on shelves (RS) stages were extracted and resolved according to Gallo *et al.* (2010). AFLP profiles were obtained from young leaves DNA following Pratta *et al.* (2011); primer combinations (PC) used in the present experiment (PC1, *MseI*31 + *EcoRI*44: *MseI*0CAC + *EcoRI*0ATC; PC2, *MseI*33 + *EcoRI*40: *MseI*0CAT + *EcoRI*0AGC; PC3, *MseI*33 + *EcoRI*46: *MseI*0CAT + *EcoRI*0ATT; PC4, *MseI*34 + *EcoRI*40: *MseI*0CTA + *EcoRI*0AGC; PC5, *MseI*34 + *EcoRI*46: *MseI*0CTA + *EcoRI*0ATT) proved to amplify random fragments in that previous report, some of them linked to the phenotypic traits.

Data analysis

The normality of phenotypic trait distributions was verified by the Shapiro-Wilk test (Pratta *et al.* 2011). For analyses at population level, the degree of genetic determination (DGD) was estimated by one-way ANOVA first considering only the 6 homozygote genotypes and then with all genotypes (the 6 homozygous and the 3 heterozygous) in order to measure the effects of hybridization on the proportion of genetic variance (Kearsey and Pooni 1996). For analyses at individual crosses level, mean values of each hybrid were compared with its respective parents by the parametric Student's *t*-test and the degree of dominance (d/a) was calculated for all fruit quality traits (Kearsey and Pooni 1996) with the aim of estimating the gene action (additive or non-additive) involved in each hybrid performance. Polypeptide and AFLP bands were assessed by presence/absence and the percentage of polymorphism was calculated by ripening stage, primer combination and overall, first in the group of RILs and then in the group of RILs and HSCs. In these analyses at a population level, *de novo* bands (those present in SCHs but not in RILs and *vice versa*) were accounted (Rodriguez *et al.* 2011) to quantify the contribution of hybridization to total molecular polymorphism. These parameters were also calculated in each cross to check the inheritance patterns of polymorphic bands. Finally, a data mining through clusters analysis was made for RILs and for RILs and SCHs, respectively, to summarize the information obtained in this experiment. The Average Euclidean distances among genotypes were calculated with all fruit quality data and the Jaccard distances among genotypes were calculated with all polypeptide and AFLP profiles data. Cluster analyses were performed through Ward's method (Kumar *et al.* 2010) and results were compared across levels of genetic variation to understand the genetic causes underlying outperforming of these SCHs.

RESULTS AND DISCUSSION

The average values, standard errors, and the corresponding d/a of all fruit quality traits are in **Table 1**, where RILs and SCHs are grouped by cross. Fruits of the different genotypes are shown in **Fig. 1**. At population level, significant DGD were found for all traits (H = 0.84, D = 0.89, S = 0.51, W = 0.87, SL = 0.42, SS = 0.92, pH = 0.97, TA = 1.00, L = 0.16, a/b = 0.20) except by F (0.00) in the group of RILs. Similar values were found when considering RILs and SCHs (H = 0.77, D = 0.89, S = 0.49, W = 0.94, SL = 0.47, SS = 0.89, pH = 0.93, TA = 0.96, L = 0.15, a/b = 0.21) though DGD was also significant for F (0.22) in this case. Hence hybridization had no effect on increasing the proportion of genetic variance of fruit quality traits in the whole population, excluding F.

Complete and partial dominance was the prevalent gene action for W, H and D, excluding TOUNR15xTOUNR9 in

Table 1 Degrees of dominance in the three analysed crosses of the fuit traits evaluated in six recombinant inbred lines and three second cycle hybrids of tomato.

Crosses	H	D	S	W	SL	SS
TOUNR1	2.85 ± 0.09 a	3.55 ± 0.06 a	0.81 ± 0.02 a	21.66 ± 1.35 a	24.79 ± 1.18 a	5.54 ± 0.08 a
TOUNR5	2.29 ± 0.08 b	2.55 ± 0.09 b	0.91 ± 0.02 b	9.84 ± 0.84 b	16.65 ± 1.57 b	6.91 ± 0.10 b
TOUNR1xTOUNR5	2.24 ± 0.11 b	2.67 ± 0.14 b	0.84 ± 0.02 a	10.54 ± 1.60 b	25.52 ± 2.14 a	7.14 ± 0.14 b
d/a	-1	-1	-1	-1	1	1
TOUNR15	2.22 ± 0.07 b	2.84 ± 0.09 c	0.79 ± 0.02 a	11.59 ± 1.04 c	14.41 ± 1.49 a	7.91 ± 0.11 b
TOUNR9	1.63 ± 0.04 a	1.83 ± 0.06 a	0.89 ± 0.01 b	3.81 ± 0.32 a	18.72 ± 0.43 b	7.04 ± 0.06 a
TOUNR15xTOUNR9	2.11 ± 0.03 b	2.36 ± 0.04 b	0.91 ± 0.01 b	7.37 ± 0.32 b	21.42 ± 0.84 b	7.20 ± 0.07 a
d/a	1	0	1	0	1	-1
TOUNR18	2.77 ± 0.07 a	2.83 ± 0.06 a	0.97 ± 0.02 a	13.72 ± 0.72 a	21.11 ± 1.20 a	7.69 ± 0.08 a
TOUNR6	1.62 ± 0.07 b	1.81 ± 0.07 b	0.91 ± 0.02 b	3.52 ± 0.41 b	18.46 ± 1.05 a	7.84 ± 0.06 a
TOUNR18xTOUNR6	1.98 ± 0.05 c	2.06 ± 0.04 c	0.97 ± 0.02 a	5.37 ± 0.28 c	26.05 ± 0.62 b	9.22 ± 0.20 b
d/a	-0.36	-0.5	1	-0.64	∞	∞
Crosses	**pH**	**TA**	**L**	**a/b**	**F**	
TOUNR1	4.96 ± 0.02 a	0.38 ± 0.01 a	39.81 ± 0.43 a	1.04 ± 0.04 a	54.17 ± 1.82 a	
TOUNR5	4.32 ± 0.04 b	0.52 ± 0.02 b	38.09 ± 0.29 b	1.14 ± 0.02 b	50.15 ± 1.72 a	
TOUNR1xTOUNR5	4.75 ± 0.04 c	0.36 ± 0.01 a	37.70 ± 0.17 b	1.17 ± 0.01 b	47.19 ± 1.01 a	
d/a	0.34	-1	-1	1	nc	
TOUNR15	4.61 ± 0.05 c	0.44 ± 0.01 a	38.81 ± 0.35 a	1.12 ± 0.03 a	54.32 ± 0.96 a	
TOUNR9	4.35 ± 0.02 a	0.80 ± 0.02 c	37.09 ± 0.07 b	1.37 ± 0.01 b	53.39 ± 0.61 a	
TOUNR15xTOUNR9	4.50 ± 0.04 b	0.55 ± 0.02 b	36.29 ± 0.17 c	1.24 ± 0.02 c	43.31 ± 1.5 b	
d/a	0.15	-0.38	-1.93	0	-∞	
TOUNR18	4.72 ± 0.03 a	0.37 ± 0.01 a	36.54 ± 0.28 a	1.15 ± 0.02 a	52.47 ± 0.82 a	
TOUNR6	4.63 ± 0.02 b	0.56 ± 0.02 b	38.66 ± 0.19 b	1.22 ± 0.02 b	51.69 ± 0.86 a	
TOUNR18xTOUNR6	4.86 ± 0.05 c	0.46 ± 0.03 c	35.74 ± 0.19 a	1.24 ± 0.01 b	50.78. ± 1.05 a	
d/a	4.5	0	-1	1	nc	

Height (H, in cm), diameter (D, in cm), shape index (S, H/D), weight (W, in g), shelf life (SL, in days), soluble solids content (SS, in ºBrix), pH, titratable acidity (TA), reflectance percentage (L), chroma index (a/b) and firmness (F). d/a = degree of dominance. Different letters indicate significant differences through parametric Student's *t*-test ($p < 0.05$) among genotypes. nc: non calculated because the were no differences among parental genotypes and the hybrid

Fig. 1 Fruits of Recombinant Inbred Lines (indicated by ToUNR*X*) and Second Cycle Hybrids (indicated as F_1) at red ripe in plant stage.

which additivity was found for D and W. RIL TOUNR1 and SCHs TOUNR1×TOUNR5 and TOUNR18×TOUNR6 had the longest SL, with dominant and overdominant gene actions being involved for this trait. For SS, trait that was controlled by dominance and overdominance, the outstanding value was found in TOUNR18xTOUNR6 (9.22 ± 0.20ºBrix). On the other hand, d/a was not calculated for F in crosses TOUNR1×TOUNR5 and TOUNR18×TOUNR6 due to lack of significant differences among the hybrid and its parents. Summarizing, and as shown in **Table 1**, at individual crosses level the most frequent gene action was non-additive (d/a ≠ 0) either with complete dominance, partial dominance and overdominance. Additivity was present just in 4 cases.

Polypeptides profiles of some genotypes are shown in **Fig. 2**. At population level (data non shown), a total of 22 polypeptides at MG, 21 at B, 17 at RR and 19 at RS were detected among the 6 RILs, the percentage of polymorphism being 73, 67, 65 and 68, respectively. Considering the four stages together, 79 polypeptide bands were found with 68% averaged polymorphism. When the 3 HSCs are included, 23 polypeptides at MG, 22 at B, 18 at RR, 20 at RS and 83 in total were accounted, with percentages of polymorphism equal to 83, 73, 72, 75 and 76, respectively. This finding involves a high proportion of *de novo* bands (13, 9, 11, 10 and 11%, respectively) whose commonest characteristic is the absence in HSCs. Rocco *et al.* (2006) firstly characterized tomato proteome in two different ecotypes and reported genotype variations according to physiological processes such as redox status control, defence, stress, carbon metabolism, energy production and cellular signalling. Faurobert *et al.* (2007) compared proteome variations during pericarp development by 2D-SDSPAGE in tomato cherry genotypes and found significant differences among polypeptide expression patterns among ripening stages. Rodriguez *et al.* (2008, 2011) and Gallo *et al.* (2010)

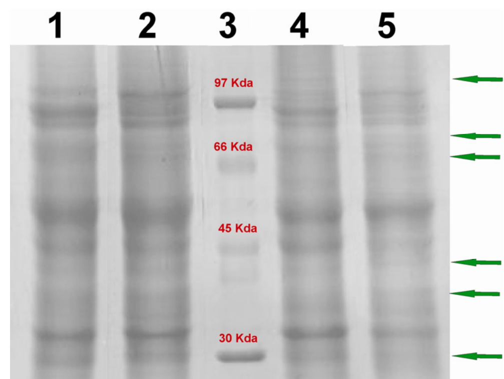

Fig. 2 Pericarp polypeptide profiles of TOUNR1 and TOUNR5 in mature green and red ripe stages. 1: TOUNR1 at red ripe stage, 2: TOUNR1 at mature green stage, 3: molecular weight marker, 4: TOUNR5 at red ripe stage, 5: TOUNR5 at mature green stage. Arrows indicate polymorphic polypeptides.

Table 2 Number of total bands (NTB), number of polymorphic bands (NPB), percentage of polymorphism (%P), number of *de novo* bands (NdnB), and percentage of *de novo* bands (%dnB) in pericarp polypeptides and AFLP profiles of the three evaluated crosses among six recombinant inbred lines of tomato.

Crosses	Pericarp polypeptides profiles														
	MG			Br			RR			RS			Total		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Parents															
NTB	17	13	17	16	15	18	14	12	16	13	15	14	60	55	65
NPB	4	6	4	5	7	5	5	5	3	2	5	4	16	23	16
%P	24	43	24	31	47	28	36	42	19	15	33	29	27	42	25
Parents + Hybrids															
NTB	18	15	17	19	15	19	15	15	17	16	16	16	68	61	69
NPB	10	10	5	8	9	7	7	8	10	6	7	7	31	34	29
%P	56	67	29	42	60	37	47	53	59	38	44	44	54	56	42
NdnB	6	4	1	3	2	2	2	3	7	4	2	3	15	11	13
%dnB	33	27	7	16	13	11	13	20	41	25	13	19	22	18	19

Crosses	AFLP profiles																	
	PC1			PC2			PC3			PC4			PC5			Total		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Parents																		
NTB	65	74	71	74	77	76	88	87	90	124	124	120	98	89	104	462	455	475
NPB	22	27	28	32	37	36	27	27	25	28	27	21	41	38	32	156	155	37
%P	34	34	38	43	48	47	31	31	28	23	22	18	42	43	31	34	34	30
Parents + Hybrids																		
NTB	74	75	75	74	79	77	89	88	92	126	126	123	98	89	107	462	455	475
NPB	31	29	32	33	42	38	28	28	30	30	32	28	41	41	38	164	168	169
%P	42	39	43	45	53	49	32	32	32	24	25	23	42	46	36	36	37	36
NdnB	9	2	4	1	5	2	1	1	5	2	5	7	0	3	6	8	13	32
%dnB	12	3	5	1	6	3	1	1	5	2	4	6	0	3	6	2	3	7

Crosses: 1: TOUNR1xTOUNR5, 2: TOUNR15xTOUNR9, 3: TOUNR18xTOUNR6. Stage of ripening: MG: mature green, B: breaker, RR: red ripe in plant, RS: red ripe in shelves. PC1: *MseI*0CAC + *EcoRI*0ATC, PC2: *MseI*0CAT + *EcoRI*0AGC, PC3: *MseI*0CAT + *EcoRI*0ATT, PC4: *MseI*0CTA + *EcoRI*0AGC, PC5: *MseI*0CTA + E*coRI*0ATT

characterized different tomato first cycle hybrids by pericarp poplypeptide profiles and reported *de novo* bands in all heterozygous genotypes which included standard and mutant for ripening *S. lycopersicum* varieties, *S. lycopersicum* var. *cerasiforme* and *S. pimpinellifolium* germplasms. Results disaggregated by cross are in **Table 2**. It is important to note that percentage of polymorphism among genotypes within individual crosses is noticeably lower than among the whole population but *de novo* bands are higher at the cross level. This fact implies that just a few bands are strictly *de novo* in this genetic background dispersed from the original parents *S. lycopersicum* cv. Caimanta and *S. pimpinellifolium* LA722.

With respect to AFLP profiles at population level (data non shown), PC1 amplified 76 bands with 55% polymorphism in the 6 RILs, these values being 81 and 59%, 95 and 41%, 129 and 29%, 107 and 56%, and 488 and 46%, for PC2, PC3, PC4, PC5, and in total, respectively. When including the 3 SCHs, 79, 82, 96, 130, 110, and 497 were detected with PC1, PC2, PC3, PC4, PC5, and in total, respectively, the percentage of polymorphism being 58, 61, 43, 31, 60, and 49%. For AFLP, *de novo* bands (presence as well as absence) were not as relevant as for polypeptides, the corresponding percentages being 5, 2, 2, 2, 6, and 3% in PC1, PC2, PC3, PC4, PC5, and in total, respectively. Examples of AFLP profiles are shown in **Fig. 3**. Results at the individual crosses level are in **Table 2**, the general trend being similar to that observed in polypeptides (lower percentage of polymorphism but higher percentage of *de novo* bands by cross than in the whole population) although less marked. AFLP was applied to study parental contribution to hybrid progeny in *Saxifraga* spp. (Dymshakova *et al.* 2012) and Vela *et al.* (2011) detected *de novo* AFLP bands in interspecific hybrids of *Drosophila*.

Fig. 4 shows cluster analyses at the three levels of genetic variation, for RILs alone (first column) and RILs and hybrids (second column) and the cophenetic correlations. Grouping of genotypes were discrepant according to the data used for clustering, reflecting the different kind of genetic variation assessed. When considering only clusters of RILs, those achieved with phenotypic traits and AFLP profiles adequately segregated parental genotypes of each

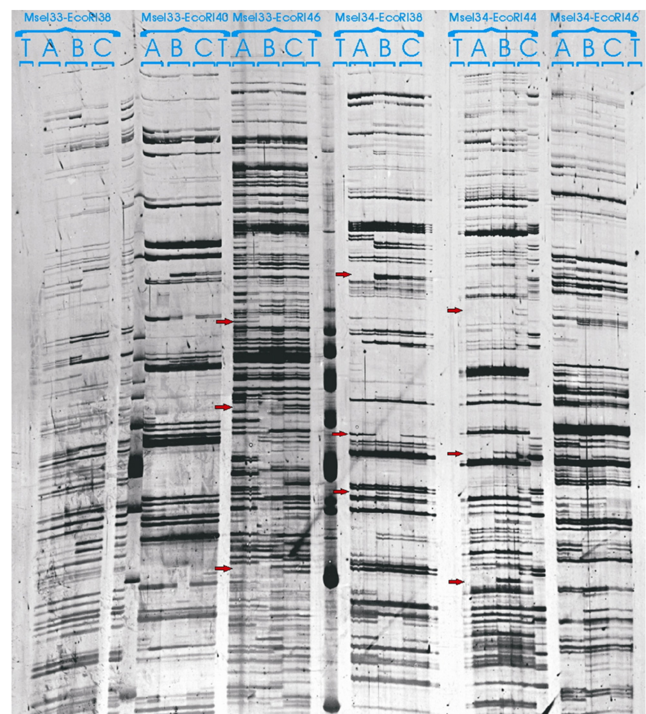

Fig. 3 AFLP profiles generated by six primers combinations in TOUNR1 (A), TOUNR5 (B), TOUNR15 (C) and a negative control (T). Arrows indicate polymorphism among RILs.

cross, this separation being clearest for the AFLP profiles given that all female parents are included in one group and the male ones in another. Instead, polypeptide profiles were not satisfactory to segregate TOUNR18 from TOUNR6, and TOUNR1 from TOUNR5.

When SCHs were included for clustering according to phenotypic traits, associations among RILs were conserved and SCHs randomly inserted into the major groups defined by RILs. This fact can be explained by the non-additive gene actions detected in this level of genetic variation, since

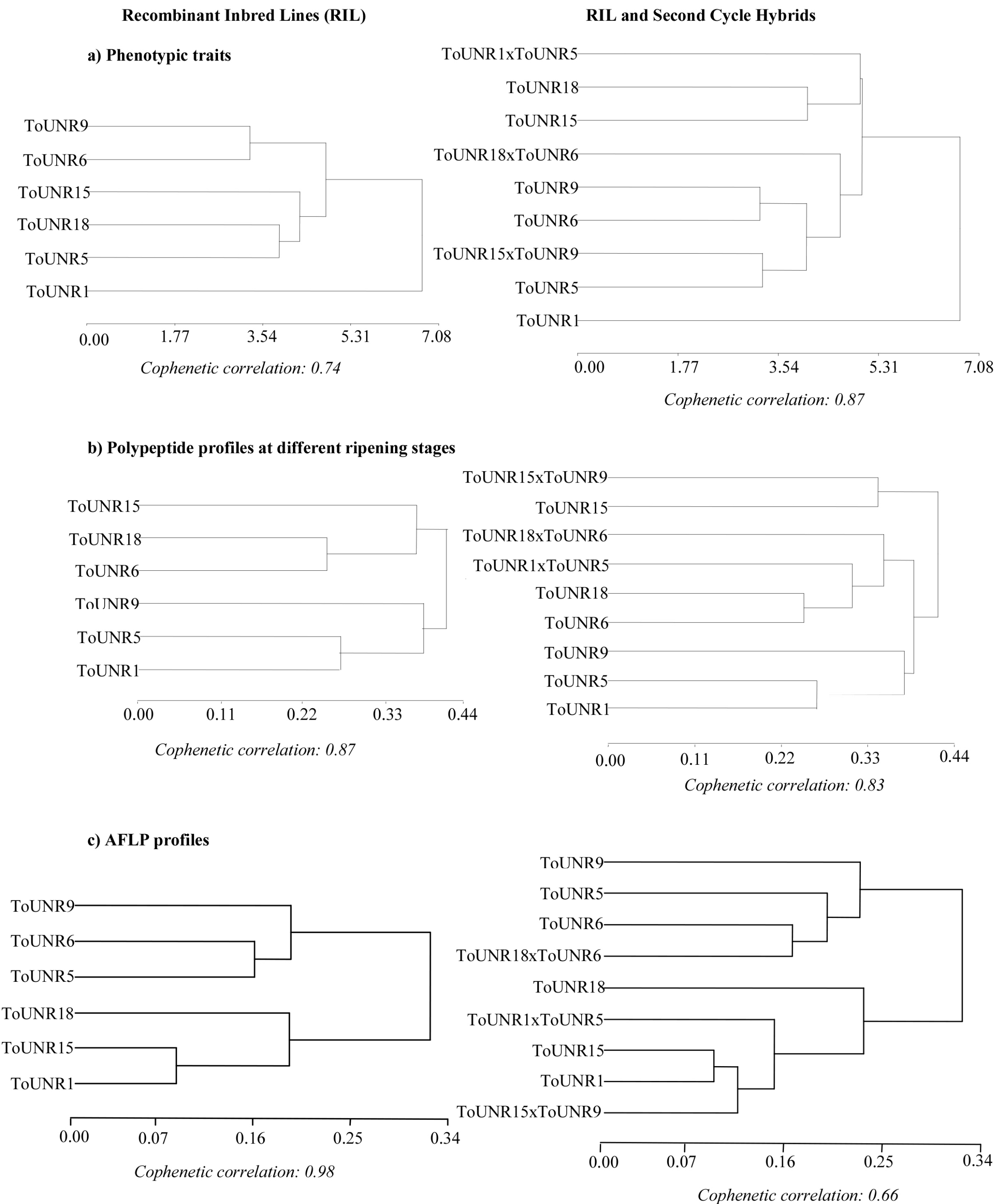

Fig. 4 Cluster analysis of the genotypes at genetic variation.

non-additivity implies that hybrid phenotype cannot be predicted from parent phenotype. Similarly, *de novo* bands were relevant for polypeptide profiles. *De novo* bands would be underlay by overdominant gene action, given that hybrid performance was different from parent performance. Accordingly, SCHs grouped rather independently from RILs in cluster from polypeptide profiles. On the other hand, and as expected since AFLP bands were mainly dominant, SCHs grouped to one of its parental RILs in the corresponding cluster.

Results from this experiment indicated that SCHs contribute with extreme values for some fruit quality traits such as shelf life, soluble solids content and firmness, in agreement with significant overdominance found in some crosses for these traits. Polypeptide characterization detected polymorphism with a similar performance to variability observed at the phenotypic level since the prevalent non-additive gene actions involved in quantitative traits could be associated to *de novo* bands revealed by SDS-PAGE. In fact, in both levels of genetic variation hybrids showed an exclusive performance, unpredictable from those of their parents.

On the other hand, AFLP bands evidenced to perform in a different way given that similar percentage of polymorphism was detected in RILs and in SCHs, and *de novo* bands were null in some primer combination and scarce in most cases. However, this molecular tool was the most efficient in separating RILs according to their parental roles in crosses and in grouping SCHs with one of its parents.

CONCLUSIONS

Characterization of tomato Second Cycle Hybrids showed different association among them and their parents according to the level of genetic variation under study (phenotypic attributes, polypeptides, and DNA), evidencing that non-additive gene action is the biological basis underlying the their outperformance.

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