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Production of New Varieties of Tomatoes Based on Yield and Fruit Quality Characters Using Molecular and Classical Breeding Techniques

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ABSTRACT. *Tomato (Solanum lycopersicum) is one of the most important vegetable crops in Sri Lanka, having a year round demand. A classical breeding programme using the Diallel crossing design produced several high yielding hybrids of tomatoes from which the best hybrid was selected to produce Recombinant Inbred Lines (RILs) superior for yield and fruit quality characters. Molecular analysis of parental and F_1 hybrid DNA using microsatellite markers proved the true hybridity of the F_1 hybrid which was used to produce the RILs. The parents V2 and V7 used to produce the F_1 hybrid were clustered in two different groups with microsatellite and Random Amplified Polymorphic DNA (RAPD) markers, showing that they were genetically distant. Hence, the product showed high heterosis or hybrid vigour.*

The hybrid was selfed up to the F_6 generation by single seed descent procedure to produce 217 RILs which were evaluated in the field in order to select lines superior for yield, high fruit number, large size of fruits, low pH, high Brix and high acidity, being parameters used for selecting varieties for cooking in local curries.

For yield, fourteen RILs performed better than the F_1 hybrid. However, a single RIL was not superior for all the traits studied. Using the statistical tool Index Method of Selection, the RILs 62, 110, 178, 186 and 36 were selected as the best new pure line varieties of tomatoes superior for yield and fruit quality traits. All these pure lines showed total field resistance to wilt disease.

INTRODUCTION

Tomato (*Solanum lycoperscicum* syn. *Lycopersicon lycopersicum, Lycopersicon esculentum* Mill*)* is the country's most important vegetable crop, both in terms of hectarage (6640 ha) and volume of production (9192 kg/ha) (Annon http://faostat.fao.org). The demand for this crop is year-round due to the versatility of its usage in both fresh (mainly in local curries) and processed food preparations.

Traditional farming can only yield a limited biomass. Better management practices and increase in acreage can lead to increased yield, but only to a limited extent. Plant breeding as

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a technology has helped increase yields in tomato to a very large extent through the judicious 'mixing' of superior alleles, whilst also conserving fruit qualities.

Classical plant breeding involves crossing or hybridization of pure lines, followed by selection to produce plants with desirable traits of higher yield, nutrition and resistance to diseases. With advancements in genetics, molecular biology and tissue culture, plant breeding is now increasingly being carried out by using molecular genetic tools as well.

A key issue in using hybridization to create new variation is the selection of the parents. The selected plants are used in the process of hybridization in order to create hybrids with heterosis or hybrid vigour (heterobeltiosis). Cultivar and hybrid development programs have relied primarily on genetic variation created at the intra-species level (Baenziger *et al,*. 2006). It is important to establish true hybridity of the progeny of a cross, especially in an inbreeding species such as tomato, as well as to establish the fact that superior hybrids can be produced by crossing parents who are genetically wide apart. Current molecular techniques enable scientists to carry out both these tests (Weerasinghe *et al*., 2004).

Whereas dominance is made use of in producing hybrids, additive gene effects are combined in producing inbred/pure lines. Pure lines are usually produced by first making a cross to produce the F_1 hybrid and thereafter selfing the hybrid to produce recombinant inbred lines (RILs). Selection and testing of superior recombinants consist of selecting, those plants that have the desired character combination among the progeny of the hybrids. The selection process is crucial to the success of the breeding objective and requires careful scientific evaluation of the progeny. This yields plants that are superior to the hybrid as well as both parents. These are self-pollinated for several generations till they reach a state of homozygosity so that the characters will not segregate in the progeny.

Baenziger *et al.,* (2006) explained that in self-pollinated crops, the value of early generation testing is ambiguous. Selfing is natural, and very efficient breeding methods exist for rapid or inexpensive inbreeding, such as bulk breeding or single-seed descent (Williams, 1981). Brim (1966) explained that a method devised for advancing generations to the desired level of inbreeding in soybeans has been in use for several years. Essentially, the method consists of advancing each F_2 plant in the population by single seed descent. In the F_2 and succeeding generations only one seed is used from each plant in the population selected as a parent for the next generation. For practical reasons, a single fruit (2-3 seeds) is taken from each plant, but only one plant from the fruit serves as the parental material.

Brim (1966) explained further that there are several advantages in this method compared to the pedigree system of breeding, such as less space requirement, considerably less time and effort in harvesting, reduction of book keeping since no records are maintained, and effective practice on selection for characters of high heritability. However, he pointed out disadvantages such as ineffectiveness of selection for characters with low heritability on a single plant basis and the loss of the identity of superior F_2 plants which cannot be recovered

Newly selected lines are evaluated for their yield and other agronomic traits of quality, and disease resistance. This evaluation is done by growing these in the research fields and recording their performance under ideal fertilizer application, irrigation, and other crop management practices.

Although choosing the type of selection unit is an important component of selection, true selection for many plant breeders is represented by the selection criteria and methods they use to choose the best among the selection units. Fundamental to understanding how to improve selection efficiency are the concepts of genetic gain and heritability. Broad sense heritability is the genotypic variance divided by the phenotypic variance. Narrow sense heritability is the additive genetic variance (the genotypic variance is due to additive, dominance, and epistatic genetic variance) divided by the total phenotypic variance. Any tool or procedure that can reduce the environmental or unknown (error) variances relative to the genetic variance of the phenotypic variance will increase heritability and the gain from selection (Baenziger *et al.,* 2006).

The considerable efforts of tomato breeders have laid emphasis mainly on yield, fruit size, fruit appearance (lack of defects and attractive color), disease resistance and more recently fruit firmness and shelf life (Salliba – Colombani *et al,*. 2001). High total soluble solids are of interest to tomato breeders because it is an important fruit quality parameter for both fresh market sales and the processing of tomatoes (Paran *et al*., 1995).

The production of RILs has several advantages over other populations in breeding programmes. As the lines are genetically homozygous, they can be propagated without further segregation and the lines can be replicated. This characteristic is particularly useful as this generation can be used as a mapping population to identify molecular markers for QTLs (Quantitative Trait Loci).

In this research, the construction of a population of RILs in tomato is described in order to select lines superior for yield and fruit quality traits as new varieties especially for use in local curries. Molecular analysis of parental and F_1 hybrid DNA was carried out to test for true hybridity of the F_1, to test the genetic distances between parents, and to identify primers that produce polymorphisms between the parents so that they could be used later for identifying molecular markers for important QTLs.

MATERIALS AND METHODS

Plant material

The RILs were constructed from an intra-specific cross between two cultivated tomato varieties Ravi and T245. This hybrid was selected among several superior hybrids produced through a previous Diallel crossing programme (Alwis *et al*., 2005). The best F_1 hybrid from the Diallel was selected for the selfing series. A total of 217 F_2 plants were self pollinated and advanced to the F_6 generation using single seed descent method, each line being derived from different F_2 plants.

Evaluation of the RILs

The 217 RILs, two parental lines and their F_1 hybrid were grown in the field at Meewatura Farm of the Faculty of Agriculture, University of the Peradeniya in a trial using the Randomized Complete Block design. A plot of four F_6 plants represented each RIL.
Observations were recorded from four F_6 plants in each plot. Fruits were harvested in bulk from the four plants of each plot. Physical and chemical traits measured were yield, fruit

weight, fruit size (length/width), titratable acidity, Brix (soluble solids content) and pH of the fruit. Chemical analyses were carried out using juice derived by blending the fruits. For each measurement for different traits, five fruits were taken from each plot. Field evaluation for resistance/tolerance to bacterial wilt was carried out throughout the trial.

DNA extraction and PCR amplification

DNA was extracted from a bulk of ten individuals from each parental line and F_1 hybrid using modified CTAB method (Federick, 1996).

Random Amplified Polymorphic DNA (RAPD) analysis

Operon primers were used as random primers in PCR to identify polymorphisms in eight parental lines used for making F_1 hybrids (Alwis *et al*., 2005). The 10-mers used as random primers in the PCR were purchased from Operon Technologies. Of a total of hundred 10-mers, the 14 primers that gave polymorphic DNA bands are listed in Table 1.

Table 1. Sequence of 10-mer Operon RAPD Primers which showed polymorphism

10-mer Primer	Sequence	10-mer Primer	Sequence
OPA1	CAGGCCCTTC	OPK13	GGTTGTACCC
OPA8	GTGACGTAGG	OPK14	CCCGCTACAC
OPA12	TCGGCGATAG	OPM15	GACCTACCAC
OPD2	GGACCCAACC	OPM16	GTAACCAGCC
OPD8	GTGTGCCCCA	OPAN6	GGGAACCCGT
OPK10	GTGCAACGTG	OPAN11	GTCCATGCAG
OPK11	AATGCCCCAG	OPAN15	TGATGCCGCT

For each RAPD-PCR reaction, 5 ng of genomic DNA was amplified with 1.2 µM of DNA primer. In a final volume of 20 µl polymerase chain reaction, 1X buffer (10 mM TRIS-HCl pH 8.2, 50 mM KCl), 2.5 mM of $MgCl_2$, 0.8 mM of each dNTPs and 0.1 U of *Taq* DNA polymerase were included. PCR reaction was performed in the Techne thermocycler. An initial denaturation of 1 min at 94 ºC was followed by 40 cycles consisting of denaturation at 93 ºC for 1 min, annealing at 35 ºC for 3 min and extension at 72 ºC for 2 min. A final cycle of extension at 72 ºC for 10 min was performed. Amplification products were separated and analyzed using 1.5% agarose electrophoresis gels at 5 V/cm.

Microsatellite analysis

Microsatellite primers of tomato were also used in PCR to identify polymorphisms between the parents as well as to test for true hybridity of the hybrid. The four microsatellite primers that gave polymorphic banding patterns among the eight parental lines are listed in Table 2.

For each Microsatellite-PCR reaction, 5 ng of genomic DNA was incubated with 0.6 µM of each of the forward and reverse DNA primers. In a final volume of 20 µl polymerase chain reaction, 1X buffer (10 mM TRIS-HCl pH 8.2, 50 mM KCl), 2.5 mM of $MgCl_2$, 0.4 mM of each dNTPs and 0.1 U of *Taq* DNA polymerase were included. The PCR reaction was

performed in the Techne thermocycler. In the touch down PCR programme, an initial denaturation of 2 min at 94 ºC was followed by 5 cycles consisting of denaturation at 94 ºC for 30S, annealing at 70 ºC for 30S and extension at 72 ºC for 30S. Five sets of 5 cycles were performed and in each set of 5 cycles, annealing temperature was reduced by 2 ºC from 70 ºC to 58 ºC in the final set. This was followed by 30 cycles consisting of denaturation at 94 ºC for 45S, annealing at 55 ºC for 45S and extension at 72 ºC for 1 min 45S. A final cycle of annealing at 55 ºC for 45S and extension at 72 ºC for 3 min was performed. Amplification products were separated and analyzed using 2% agarose gels at 5 V/cm and 10% polyacrylamide gels.

Table 2. Sequence of microsatellite primers which showed polymorphism

Primer	Sequence 5'	Repeat Sequence	Fragment size
TMM3	GGATTGTAGAGGTGTTGTIGG TTTGTAATTGACTTTGTCGAIG	$(GT)_{32}(AT)_{67}$	412
TMM6	CTGTTTACTTCAAGAAGGCTG ACTTTAACTTTATTATTGCCACG	$(TAT)_{15}(TGT)_4$	166
TMM7	CATTTTATCATTTATTTGTGTCTTG ACAAAAAAAGGTGACGATACA	$(TA)_2(TAT)_9$	104
TMM9	GGTGATAATTTGGGAGGTTAC CGTAACAGGATGTGCTATAGG	$(AAG)_6TT(GAT)_7$	105
TMM10	AACATTAGTTTGATTGGATGG TTAAACTTTGCTTGACTTTCC	$(C)_{16}$	335

Statistical analysis

Phenotypic evaluations of RILs were done using the iMAS computer programme and in the analysis of DNA profiles, presence of clear bands representing alleles of each locus was scored as '1' and absence of bands scored as '0' (Weerasinghe *et al*., 2004). The data were statistically analyzed using the SPSS version 10 software package.

RESULTS AND DISCUSSION

Confirmation of true hybridity

The true hybridity of the F_1 hybrid was tested and confirmed by the use of microsatellite markers as shown in the Plates 1 and 2. The two allelic bands of the parents and the presence of both bands in the hybrid confirmed true hybridity.

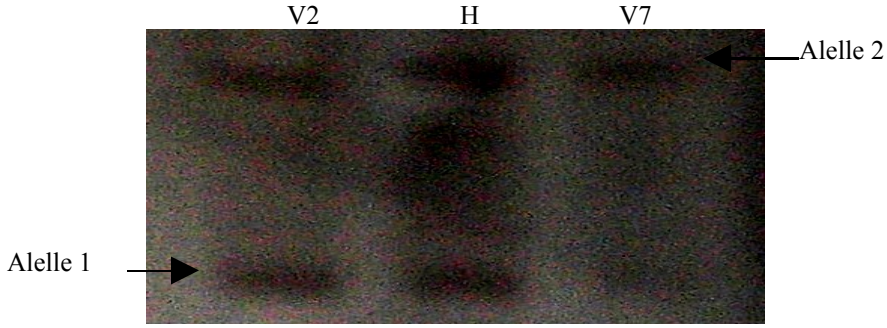

Plate 1. DNA profile in 10% polyacrylamide gel obtained from microsatellite primer TMM10 with two parents and the hybrid (H: Hybrid and V2, V7: Parents)

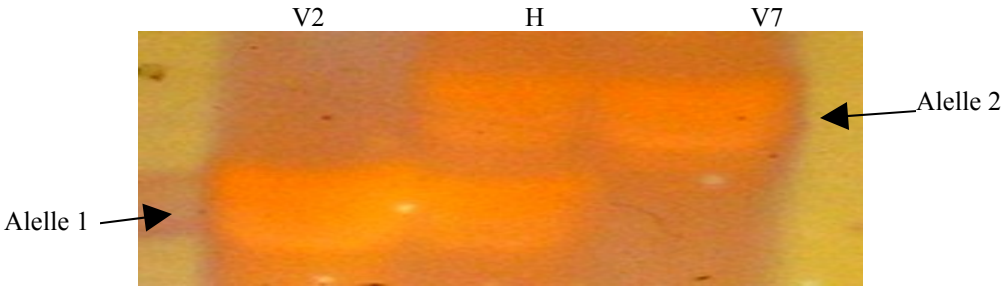

Plate 2. DNA profile in 10% polyacrylamide gel obtained from microsatellite primer TMM6 with two parents and the hybrid (H: Hybrid and V2, V7: Parents)

Apart from the DNA band present in the female parent, the band belonging to the male parent appeared in all the hybrids as expected (Weerasinghe *et al*., 2004 and Marshall *et al.*, 1994).

Characterization of parental tomato varieties with RAPDs markers

Genetic divergence is one of the useful tools for selection of parents for hybridization to develop high yielding varieties. Inclusion of more diverse parents in hybridization is believed to increase the chances of obtaining stronger heterosis and gives a broad spectrum of variability in segregating generations. The degree of divergence among the biological population at intra and inter cluster levels permits the selection of genetically diverse parents to obtain the desirable recombinants in the segregating generations upon crossing. It may be possible to obtain heterotic segregants if the varieties / lines of one cluster are crossed with the varieties / lines of another cluster. The parents separated by the medium magnitude of divergence generally show higher heterosis (Akter *et al*., 2002).

For analyzing the genetic diversity of a population, the PCR based RAPDs technique can be effectively used when it is properly optimized. First, the selection of the random primers with high polymorphism is very important.

Plate 3 shows PCR amplification products of DNA of eight parental tomato varieties using three Operon primers OPK 10, OPK 11 and OPK 14, showing clear polymorphisms.

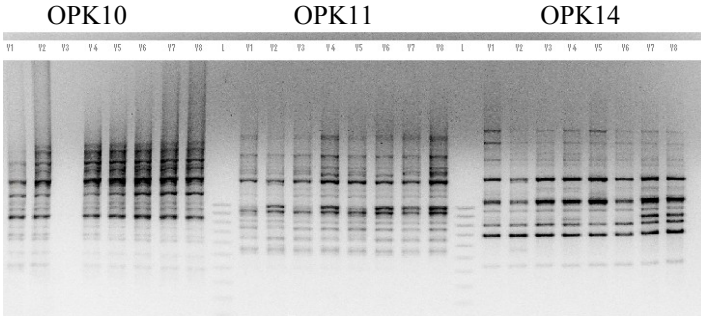

Plate 3. RAPD profile in 1.5% agarose gel obtained from three different Operon random primers (OPK10, OPK11 and OPK14).

(1-8 parental tomato varieties with OPK10 primer, 10-17 parental tomato varieties with OPK11 primer, 18-25 parental tomato varieties with OPK14 primer)

The 14 random primers produced 101 scorable polymorphic RAPDs bands among the eight parents. The polymorphisms produced by using random primers were statistically analyzed to obtain the dendogram as shown in Figure 1.

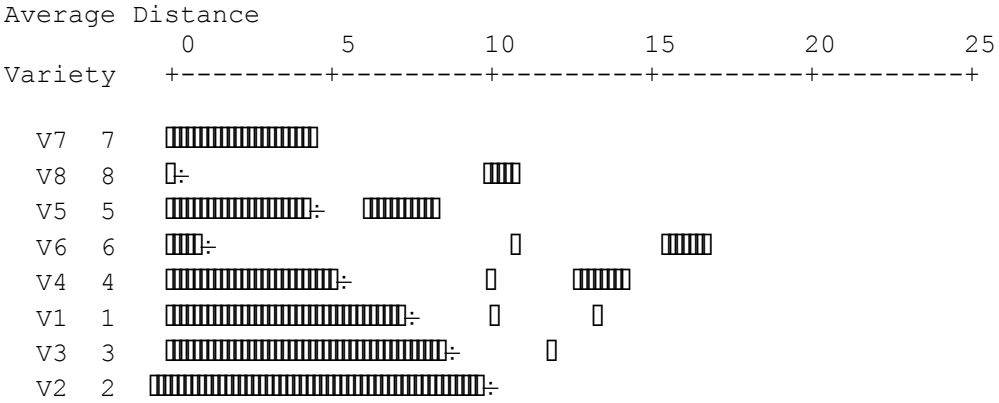

Figure 1. The dendogram obtained for 8 parental tomato varieties using 14 Operon random primers.

According to the dendogram obtained from the SPSS analysis, eight varieties can be grouped into 4 clusters, where five varieties V4, V5, V6, V7 and V8 were in one group at average distance of 13 and three other varieties V1, V2 and V3 clustered in three individual groups at average distance of 18, 20 and 25 respectively.

The parents V2 and V7 used to produce the F_1 hybrid were clustered in two different groups showing that they were genetically distant and hence the product showed high heterosis or hybrid vigour (Figure 1).

Characterization of parental tomato varieties with Microsatellite markers

Eight parental tomato varieties were tested using ten microsatellite primers of tomato. Plates 4 and 5 show the DNA profiles of the amplified products of microsatellite primers TMM7, TMM9 and TMM10.

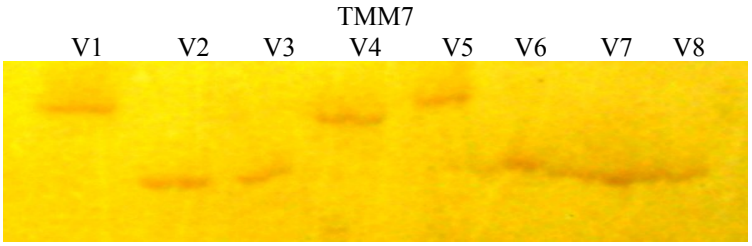

Plate 4. DNA profile in 10% polyacrylamide gel obtained from microsatellite primer TMM7 (V1 – V8 represent eight parental tomato varieties)

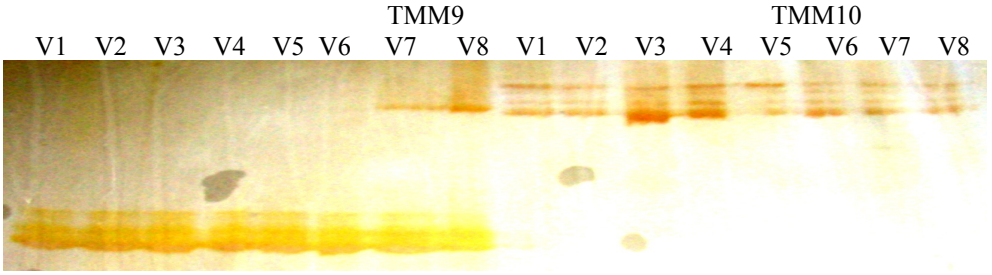

Plate 5. DNA profile in 10% polyacrylamide gel obtained from microsatellite primers TMM9 & TMM10

(1-8 parental tomato varieties (V1 – V8) with microsatellite primer TMM9, 9-16 parental tomato varieties (V1 – V8) with microsatellite primer TMM10)

The dendogram produced by analyzing the polymorphisms using SPSS statistical software is shown in Figure 2.

Average Distance

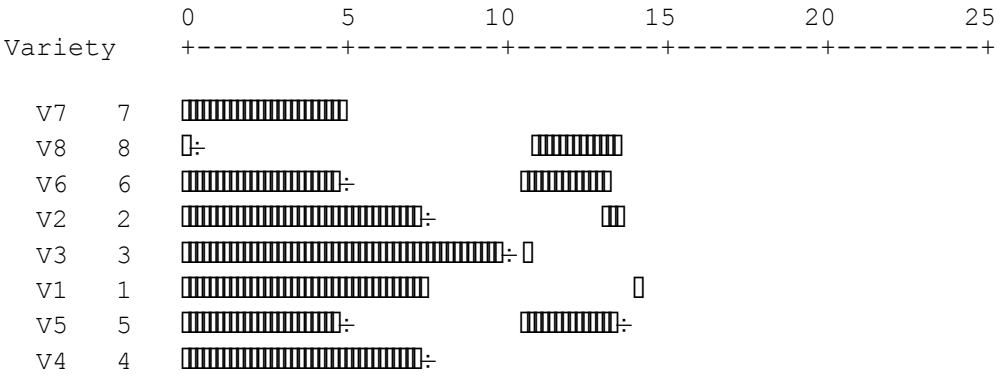

Figure 2. The dendogram obtained for 8 tomato varieties with five microsatellite primers.

At the average distance of 15, five different groups of eight varieties can be observed where V6, V7 & V8 are clustered in one group at the average distance of 12, V2 clustered in one group at the average distance of 18, V3 clusterd at the average distance of 24, V1 and V5 clustered in one group at average distance of 12 and V4 clustered in one group at the average distance of 18. The varieties V2 & V7 used as parents in the production of the F_1 hybrid are clustered in two different groups. This again confirms the reasons for getting high heterosis when parents are distantly placed in the clusters. Kalinowski (2002) showed that highly polymorphic loci of microsatellite provided better estimates of genetic distances than less polymorphic loci and the requirement of a sufficient number of alleles be examined for estimating genetic distances.

Evaluation of RILs / pure lines

Accordingly, with all this information, the F_1 hybrid (V_2 x V_7) was selfed to produce F_6 RILs using SSD procedure, since this method maintains a reasonable genetic base even for selection among F_6 SSD lines for characteristics of lower heritability and SSD offers greatest benefits in situations where simultaneous selection is required for several characteristics under different heritabilities (Casali *et al*., 1975a & b and Fahim *et al*., 1998). Selection was for high yield, high fruit number, large size of fruits, low pH, high Brix and high acidity as selection was for tomatoes to be used in cooking local curries.

Table 3 shows the RILs that performed best for each trait separately.

In considering yield, fourteen RILs performed better than the F_1 hybrid of which RIL 62 gave the highest yield of 1675 kg. It also gave the highest fruit number (33). The RIL 110 was also superior in yield (1494.5 kg) and fruit number (32). The largest fruit size (1.16) was shown by RIL 178. The highest pH of 4.57 was shown by RIL 101, while RIL 107 gave the highest Brix value (5.4) and four RILs showed similar acidity (8.0).

As expected, and as shown in Tables 3 & 4, a single RIL was not superior for all the traits. Fahim *et al*. (1998) explained that the production of lines with a superior multiple phenotype is a much more difficult task than selecting lines that excel for a single character. In such

circumstances, it is the best to use a statistical tool such as the Index method to select the best line/s.

Table 3. Best five RILs for yield and fruit quality characters

Yield		Fruit No.		Fruit Size		pH		Brix		Acid	
Best RIL	Yield (kg)	Best RIL	Fruit No.	Best RIL	Size	Best RIL	pH	Best RIL	Brix	Best RIL	Acid
62	**1675.0**	151	**33**	178	**1.16**	101	**4.57**	107	**5.4**	2	**8.00**
110	**1494.5**	62	**32**	103	**1.13**	6	**4.55**	95	**5.2**	14	**8.00**
202	**934.0**	110	**32**	170	**1.12**	210	**4.54**	166	**5.2**	81	**8.00**
237	**915.0**	237	**28**	149	**1.11**	208	**4.48**	240	**5.2**	225	**8.00**
186	**833.4**	97	**27**	138	**1.11**	238	**4.47**	130	**5.1**	248	**7.90**
Hybrid	**707.5**		**16**		**0.91**		**4.14**		**4.0**		**3.20**

Index method of selection

For construction of selection indices, estimates of certain parameters are required. These estimates being specific to a specific population, the application of selection index is thus valid to that specific population only. The Fisher's discriminant function was used in construction of selection index model.

The discriminant function $Z = b_1x_1 \times b_2x_2 \times b_3x_3 \times \ldots\ldots \times b_n x_n$
where, x_1, x_2, …. x_n are the variables measured, and b_1, b_2 ….. b_n are the weighted coefficients (Singh and Chaudhary, 1977).

Table 4 gives the means and ranks of 14 best RILs amongst 217 RILs, parents and F_1 hybrid for yield and fruit quality characters. The hybrid showed heterosis for yield and size of fruit but was ranked behind 14 RILs for yield and 12 RILs for fruit size. In fact, 14 RILs showed characters superior to the hybrid.

Therefore, in the calculation of the selection index, the selection criteria used were 40% for Yield, 10% for Fruit size, 10% for pH, 20% for Brix and 20% for Acidity. The Z score was calculated as follows.

$$Z\text{-score} = \frac{x - \mu}{\sigma}$$ where, μ = mean and σ = standard deviation

(Fowler and Cohen, 1990)

The selection index (SI) was then constructed as follows.

Selection Index (SI) = (Z-score$_{Yield}$ x 40%) + (Z-score$_{Size}$ x 10%) + (Z-score$_{pH}$ x 10%) + (Z-score$_{Brix}$ x 20%) + (Z-score$_{Acid}$ x 20%)

Table 5 shows the SI scores and the respective ranks of the 14 RILs. All of them showed total field resistance to bacterial wilt.

Table 4. Means and ranks for yield and fruit quality characters

Genotype	Yield	Rank	Fruit No.	Rank	Fruit Size	Rank	pH	Rank	Brix	Rank	Acid	Rank
P1	552.3	38	8	21	0.79	35	4.21	27	3.2	22	3.30	66
P2	383.6	89	19	10	1.06	8	4.38	11	4.0	14	2.80	73
Hybrid	707.5	15	16	13	0.91	23	4.14	34	4.0	14	3.20	68
Fourteen Best RILs compared to Hybrid												
62	1675.0	1	32	2	0.90	24	4.27	21	3.4	20	2.6	77
110	1494.5	2	32	3	0.76	38	4.31	17	4.0	14	2.55	78
202	934.0	3	17	12	0.90	24	4.23	25	3.9	15	4.55	41
237	915.0	4	28	4	0.87	27	4.11	37	3.0	24	1.70	88
186	833.4	5	18	11	0.83	31	4.17	31	4.5	9	5.95	17
18	818.8	6	21	8	0.96	18	4.24	24	4.0	14	5.30	28
106	808.3	7	15	14	0.84	30	4.24	24	3.8	16	3.55	61
108	807.8	8	15	14	0.79	35	4.12	36	4.0	14	4.60	40
19	796.3	9	22	7	0.87	27	4.28	20	3.7	17	3.20	68
36	790.0	10	19	10	0.82	32	4.17	31	4.4	10	6.50	11
151	775.0	11	33	1	1.00	14	4.22	26	4.1	13	2.65	76
82	745.0	12	17	12	0.96	18	4.39	10	4.0	14	4.80	36
178	734.2	13	21	8	1.16	1	4.16	32	4.8	6	5.30	28
105	726.5	14	22	7	0.97	17	4.25	23	3.9	15	3.40	64

Selection of new varieties of pure lines of tomatoes

According to the selection index (SI), the RILs 62, 110, 178, 186 and 36 can be selected as the best new pure line varieties of tomatoes for possible cultivation on a commercial scale.

CONCLUSIONS

Five superior pure lines of tomatoes were selected as new varieties for commercial production in Sri Lanka. They were specially selected for superiority in yield and fruit quality characters being most suitable for use in the preparation of local curries. Molecular markers (RAPDs and SSRs) were used to test the success of the hybridization technique as well as to infer the cause of high heterosis. Fourteen RILs showed traits superior to the hybrid and the parents. All of these showed field resistance to bacterial wilt.

In the selection of the best RILs as the new varieties, the selection index method was used, whereby the RILs 62, 110, 178, 186 and 36 were selected as new improved varieties of tomatoes for commercial production in Sri Lanka.

Table 5. Selection Indices of the best 14 RILs

Genotype	Selection Index	Rank	Genotype	Selection Index	Rank
62	1.27	1	108	0.42	11
110	1.13	2	19	0.31	13
202	0.72	7	36	0.80	5
237	-0.02	14	151	0.47	9
186	0.84	4	82	0.68	8
18	0.77	6	178	1.06	3
106	0.38	12	105	0.43	10

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