

Research Paper

Mapping of fruit length related QTLs in interspecific cross (*Capsicum annuum* L. × *Capsicum galapagoense* Hunz.) of chilli

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Fruit length in chilli is quantitatively inherited trait and selection based on phenotypic performance is tedious and time consuming. To detect QTLs determining fruit length in *Capsicum* spp., an interspecific F₂ mapping population was developed from the cross of *C. annuum* L. cv. 'FL 201' with *C. galapagoense* Hunz. accession 'TC 07245'. Fruit length in this cross showed a quantitative inheritance with the population depicting a symmetric distribution in histogram. To map quantitative trait loci (QTLs) for fruit length 400 SSR markers were surveyed on the parental genotypes but only 28 markers were observed to be polymorphic indicating less genetic diversity between the two *Capsicum* species. Polymorphic markers were then analyzed in F₂ population consisting of 210 plants and 24 of these markers were mapped on to three linkage groups (LGs): LG 1, LG 2 and LG 3. Two fruit length determining QTLs designated as *pauff2.1* and *pauff2.2* were identified and both the QTLs were mapped on to LG 2. The two QTLs together explained 21.78 per cent of the phenotypic variation. Apart from the two QTLs, positive alleles were detected in the small fruited parent 'TC 07245' which might be of potential use in chilli breeding programs.

Key Words: chilli pepper, fruit length, molecular markers, *pauff2.1*, *pauff2.2*, QTL mapping.

Introduction

Chilli or hot pepper belongs to the genus *Capsicum* five species of which namely *C. annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L. Ruiz. & Pavon, and *C. pubescens* Ruiz. & Pavon are domesticated. *C. annuum* L. is the widely cultivated species worldwide and includes both, the chilli pepper and the bell pepper (Bosland 1992). Improving yield related traits have remained a major goal for chilli breeding (Barchi *et al.* 2009, Zygier *et al.* 2005). Fruit length in chilli determines consumer acceptability and contributes directly to the yield. This is a polygenic trait with narrow sense heritability of 0.68–0.76 (Ben Chaim *et al.* 2001). The conventional biometrical approaches, although descriptive of inheritance, but do not explain the effects of individual quantitative trait loci (QTL) affecting a trait. Therefore, breeders grow large populations over locations and years to reliably make selection on phenotypic basis. This makes the field evaluation for fruit length and yield improvement cumbersome and time consuming with

low genetic gains.

In recent years, progress in molecular marker technology has permitted breeders to identify individual QTLs and estimate their effects on phenotypic performance (Barchi *et al.* 2009, Lu *et al.* 2012). Introgression of QTLs from the related species to the commercial types has been accelerated through Marker-Assisted Breeding (MAB). Among the molecular markers, Simple Sequence Repeats (SSRs) have been extensively used in mapping programs due to their suitability for automation, high throughput and good genome coverage (Hearne *et al.* 1992, Mimura *et al.* 2012, Powell *et al.* 1996, Sugita *et al.* 2013). Despite the advances made in marker technology, progress in mapping QTLs in chilli has been rather slow when compared with other Solanaceous crops such as tomato (*Solanum lycopersicum* L.). The probable reasons could be that chilli has larger genome size, approx. 3× that of tomato (Park *et al.* 2011) and exhibit low levels of polymorphism (Dhaliwal *et al.* 2014, Dwivedi *et al.* 2013). To facilitate MAB in chilli, Barchi *et al.* (2009), Ben Chaim *et al.* (2001), Dwivedi *et al.* (2013), and Han *et al.* (2016) detected QTLs for fruit length, fruit diameter, fruit shape, fruit weight and other yield related traits in populations originating from intra- and inter-specific crosses.

The objective of this study was to identify and map QTLs for fruit length in F₂ population derived from an

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inter-specific cross of long fruited *C. annuum* cv. ‘FL 201’ × short fruited *C. galapagoense* accession ‘TC 07245’. The identified QTLs would facilitate their introgression to the commercial types through MAB and might serve as potential target regions for cloning of the candidate genes through fine mapping.

Materials and Methods

Plant material

The plant material was developed from the inter-specific cross involving long fruited *C. annuum* cv. ‘FL 201’ and short fruited *C. galapagoense* accession ‘TC 07245’. Plants of ‘FL 201’ are medium tall (50–55 cm) with light green foliage, purplish anthers and pendent fruit bearing habit. Plants of ‘TC 07245’ are tall (80–85 cm) with dark green foliage, pale green anthers and erect fruit bearing habit. The parental lines were maintained by continuous selfing for several generations. The F₁ seed was generated by controlled pollination in 2013 using ‘FL 201’ as a maternal parent. Erect fruit bearing habit, the trait inherited from the paternal parent, confirmed hybridity of the F₁ plants. The F₁ hybrid was raised in 2014 in an insect-proof cage to generate the F₂ seed. The F₂ seed were sown in October 2014 and the mapping population comprising of 210 F₂ plants along with the two parents was transplanted to the field in February 2015.

Trait evaluation

Individual F₂ plants and the two parents were phenotyped for fruit length at the second harvest. Five red ripe fruits were taken randomly from different plant positions and fruit length, the distance from the pedicel attachment to its apex, was measured in cm. Means were used for classification of fruit length following the scale described in **Table 1**.

Broad sense heritability (h^2_{bs}) of fruit length was estimated following Mahmud and Kramer (1951) as follow;

$$h^2_{bs} = VF_2 - \frac{\sqrt{(VP_1 \times VP_2)}}{VF_2}, \text{ where}$$

VF_2 = phenotypic variance of F₂, VP_1 = phenotypic variance of P₁, VP_2 = phenotypic variance of P₂, and VF_1 = phenotypic variance of F₁.

Table 1. Scale used for classification of the fruit length in F₂ population derived from the cross *C. annuum* ‘FL 201’ × *C. galapagoense* ‘TC 07245’ chilli

S.No. ¹	Fruit length (cm)	Classification
1	3.80 to 4.79	Very small fruit
2	4.80 to 6.79	Small fruit
3	6.80 to 7.79	Medium long fruit
4	7.80 to 8.79	Long fruit
5	8.80 to 10.79	Very long fruit
6	10.80 and above	Extra-long fruit

¹ S.No. = Serial Number.

SSR amplification and polymorphism

The genomic DNA was isolated from fresh leaves following the method described by Singh *et al.* (2009). DNA was quantified using spectrophotometer (Nanodrop 1000, Thermo scientific Inc., Waltham, MA USA) and adjusted to 50 ng/μl by adding TE. The PCR was performed in a reaction mixture (25 μl) containing 2.0 μl (50 ng) genomic DNA, 1.5 μl (5 μM) of each forward and reverse primers, 0.5 μl (10 mM) dNTP mix, 1.5 μl (25 mM) MgCl₂, 5 μl (5×) green flexi PCR buffer, 0.12 μl (5 U/μl) *Taq* polymerase and 12.8 μl of sterile distilled water. The PCR reagents were procured from Promega, Madison, WI, USA.

SSR marker analysis was performed to determine the polymorphism between the two parents, ‘FL 201’ and ‘TC 07245’. In total, 400 SSR markers including 112 genic (gene based SSR markers with a known location on a chromosome) and 288 genomic markers (developed from SSR enriched genomic libraries) covering the whole *Capsicum* genome were screened. The genic markers were selected from genetic maps developed by Ince *et al.* (2010), Lee *et al.* (2004), Minamiyama *et al.* (2006) and Yi *et al.* (2006). Primer sequences for genomic SSR primer pairs were received from Dr. Roland Schafleitner, the World Vegetable Center, Taiwan. List of 400 primer pairs used for parental polymorphism survey is given in **Supplemental Table 1**.

The DNA amplifications were performed in Master Cycler 5331-Eppendorf version 2.30.31-09, Germany. A touchdown PCR program was followed to amplify the DNA fragments, that is initial denaturation at 94°C for 3 min followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 60°C (the annealing temperature for each cycle being reduced by 1°C per cycle) for 1 min and extension at 72°C for 1 min, and subsequently, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products of SSR markers were first fractionated at 4.0 per cent agarose gel. The markers which did not show any detectable parental polymorphism on agarose gels were subjected to 6.0% polyacrylamide gels (PAGE) using 0.5× Tris-borate-EDTA (TBE) buffer containing ethidium bromide (HiMedia Labs. Pvt. Ltd., Mumbai India) and visualized under UV light on an Alpha Imager[®] HP imaging system (Fisher Scientific Ltd., Loughborough UK). The results were then confirmed through high resolution MCE[®]-202 multiNAMicro-chip automated electrophoresis system.

QTL analysis and genetic mapping

Means of individual F₂ plants were used for QTL analysis. MAPMARKER/EXP v3.0b program (Lander and Botstein 1989) was used to create a framework linkage map for QTL detection. The map was generated at a Logarithm of Odds (LOD) score of 3.0 and maximum recombination fraction of 30 centi Morgan (cM). Linkage distances in terms of cM were calculated by means of the Kosambi’s mapping function. The commands ‘Order’ and ‘Rip’ were used to assign the order of markers on the map. Mapping was

performed using the QTL Cartographer v2.5 (Wang *et al.* 2010). Percentage of phenotypic variation accounted for individual QTL was estimated by the coefficient of determination (R^2). Additive (a) and dominance (d) effects were determined by single-marker analysis with the highest F-value within a given QTL region. The detected QTLs were designated as per the International Rules of Genetic Nomenclature. In the assigned nomenclature, 'pau' indicated name of the institution 'Punjab Agricultural University', 'fl' indicated the trait 'fruit length', the first number indicated the pepper linkage group (LG) involved, and the second number indicated the position within the LG to which the QTL is mapped.

Results

Phenotypic evaluation of F_2 population

The phenotypic diversity in fruit length between the parental lines 'FL 201' and 'TC 07245', and a wider range within the F_2 population is depicted in Figs. 1, 2, respectively. The fruit length data of the 210 F_2 plants were classified into six categories: very small, small, medium long, long, very long and extra-long fruit length. Variation in the F_2 population is represented graphically through the frequency distribution in Fig. 3. The maximum number of segregants (29.52%) was grouped under the 'medium long' category. The frequency distribution formed nearly the symmetric histogram indicating that fruit length in chilli is quantitatively inherited trait with additive gene effects. Some segregants with mean values on either side of the parental range were also observed. Broad sense heritability (h^2_{bs}) of fruit length in F_2 population of chilli was 0.75.

Marker analysis and QTL mapping

Of the 400 SSR markers used in parental polymorphism survey, only 28 markers were polymorphic. The list of polymorphic SSR markers is given in Table 2. For genotyping, banding pattern obtained across 210 F_2 population was scored and marker segregation pattern was checked for their

fitness to the expected 1:2:1 (AA:AB:BB) genetic ratio. The observed χ^2 values for all the polymorphic markers are less than the tabulated χ^2 value 5.99 at 5 per cent level of significance and 2 degree of freedom. Thus, genotypic segregation data fitted well and do not deviate from the expected genotypic ratio. Segregation pattern of selected SSR markers resolved on agarose and PAGE are shown in Fig. 4a, 4b, respectively.

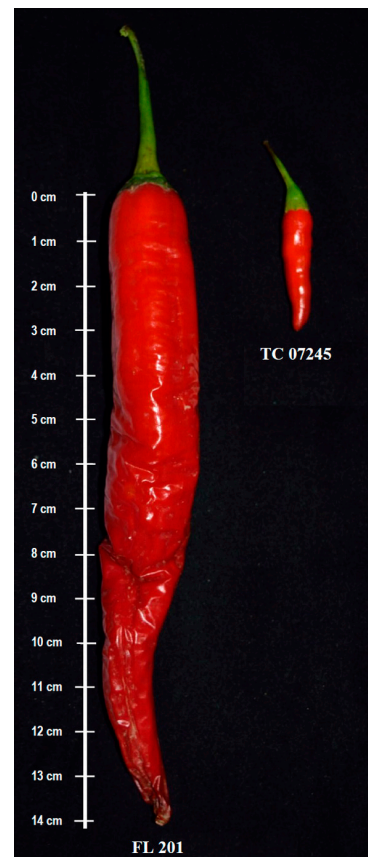


Fig. 1. Phenotypic variability for fruit length between parental lines 'FL 201' and 'TC 07245'.

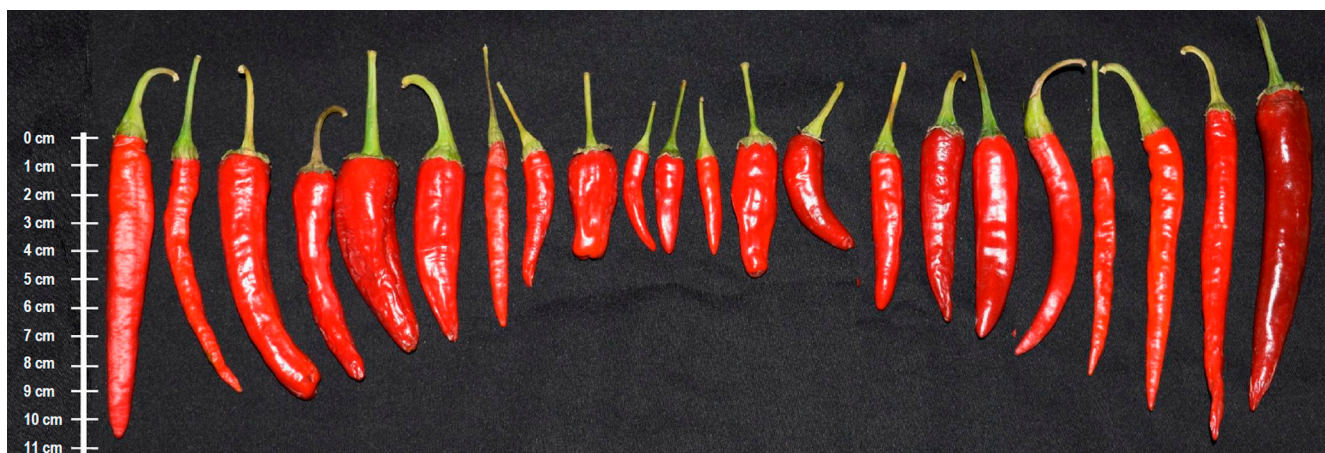


Fig. 2. Phenotypic variation for fruit length in F_2 population derived from the cross *C. annuum* 'FL 201' \times *C. galapagoense* 'TC 07245' chilli.

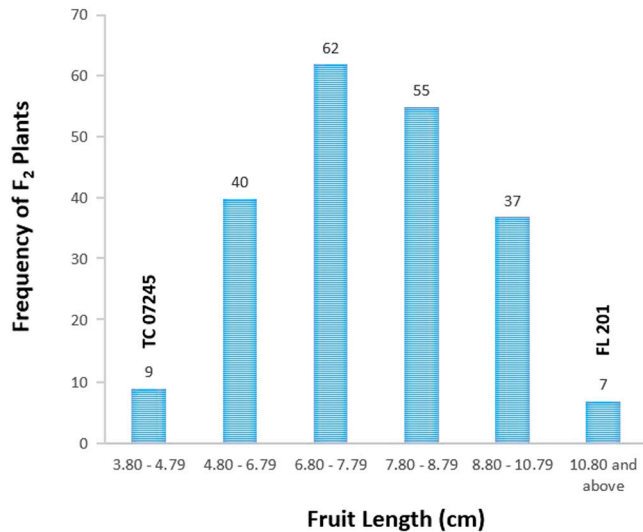


Fig. 3. Frequency distribution for fruit length in 210 F₂ population derived from the cross *C. annuum*. ‘FL 201’ × *C. galapagoense* ‘TC 07245’.

The genotypic data of polymorphic markers were used for assigning linkage positions of the markers with the help of MAPMAKER/EXP 3.0b. Of the 28 polymorphic SSR markers scored, map position of 24 markers was established

as shown in **Fig. 5**. Linkage analysis mapped 12 markers on to LG-1, 8 markers on to LG-2, and 4 markers on to LG-3. The remaining 4 markers could not be linked to any of the Linkage groups. The genetic map covered a total map length of 399 cM with an average marker interval of 16.62 cM between two markers. Based on the BLAST analysis, position of the unlinked markers AVRDC-PP161, AVRDC*MD911, CAMS 684 and Hpms 2-9 was located on the chromosome 12, 1, 6 and 3, respectively.

Marker analysis following composite interval mapping (CIM) of the F₂ population detected two QTLs determining fruit length. The QTLs designated as *pauffl2.1* and *pauffl2.2* were conditioned by the marker pairs AVRDC-PP236-AVRDC*MD 705 and GPMS 100-AVRDC*MD 782 (**Fig. 5**). Both the QTLs were mapped on to the LG-2. The QTL likelihood plots of LG-2 showing the two LOD peaks for fruit length in F₂ population are shown in **Fig. 6**. The QTL *pauffl2.1* was found to be the major QTL explaining 13.38 per cent of the phenotypic variation with LOD score of 5.53. The minor QTL *pauffl2.2* explained 8.40 per cent of the phenotypic variation with LOD score of 5.26. The identified QTLs showed positive additive effects and were derived from the long fruited ‘FL 201’. The F₂ population mean for the four markers *viz.*, AVRDC-PP236, AVRDC*MD705, GPMS 100 and AVRDC*MD782 spanning QTLs *Pauffl2.1* and *Pauffl2.2* respectively were analyzed. At marker locus,

Table 2. Test of significance for segregation of polymorphic SSR markers in the F₂ population derived from the cross FL 201 × TC 07245

SSR primer	Plant population scored	Observed frequency values*				Expected frequency values				χ^2 (1:2:1)	P-value at P ≤ 0.01
		P1 allele	H allele	P2 allele	Not amplified	P1 allele	H allele	P2 allele	Ratio		
AF244121	210	48	110	52		52.5	105	52.5	1:2:1	0.628	0.730
AF130118	210	47	110	53		52.5	105	52.5	1:2:1	0.819	0.664
AVRDC*MD711	210	54	113	43		52.5	105	52.5	1:2:1	2.372	0.305
AVRDC-PP231	210	47	112	51		52.5	105	52.5	1:2:1	1.086	0.581
AVRDC*MD678	210	52	110	48		52.5	105	52.5	1:2:1	0.874	0.646
AVRDC-PP124	210	54	112	44	2	52	104	52	1:2:1	1.774	0.412
CAMS 844	210	48	109	53		52.5	105	52.5	1:2:1	0.543	0.762
AVRDC-PP171	210	51	111	58		52.5	105	52.5	1:2:1	0.771	0.680
AVRDC-PP102	210	47	113	50		52.5	105	52.5	1:2:1	1.306	0.520
HpmsE063	210	49	111	50	2	52	104	52	1:2:1	0.600	0.740
HpmsE028	210	52	108	50		52.5	105	52.5	1:2:1	0.210	0.900
HpmsE062	210	46	110	54	2	52	104	52	1:2:1	1.115	0.573
CAMS 888	210	48	111	51		52.5	105	52.5	1:2:1	0.773	0.679
AVRDC-PP250	210	48	113	49		52.5	105	52.5	1:2:1	1.230	0.541
AVRDC-PP236	210	50	112	48		52.5	105	52.5	1:2:1	0.973	0.615
AVRDC*MD705	210	48	109	53		52.5	105	52.5	1:2:1	0.542	0.763
GPMS 100	210	54	110	46		52.5	105	52.5	1:2:1	0.967	0.617
AVRDC*MD860	210	43	112	53	2	52	104	52	1:2:1	2.250	0.325
AVRDC*MD782	210	51	110	49		52.5	105	52.5	1:2:1	0.514	0.773
CAMS 808	210	49	109	52		52.5	105	52.5	1:2:1	0.391	0.822
AVRDC-PP711	210	45	114	51		52.5	105	52.5	1:2:1	1.885	0.390
AVRDC-PP116	210	50	112	48		52.5	105	52.5	1:2:1	0.972	0.615
HpmsE119	210	53	113	44	2	52	104	52	1:2:1	1.643	0.440
HpmsE010	210	52	109	49		52.5	105	52.5	1:2:1	0.390	0.823
Hpms 2-9	210	48	111	51		52.5	105	52.5	1:2:1	0.772	0.680
AVRDC-PP161	210	49	114	47		52.5	105	52.5	1:2:1	1.580	0.454
AVRDC*MD911	210	53	112	45		52.5	105	52.5	1:2:1	1.543	0.462
CAMS 684	210	46	113	51		52.5	105	52.5	1:2:1	1.456	0.483

* Where: P1, allele from FL 201; H, Heterozygous allele; P2, allele from TC 07245.

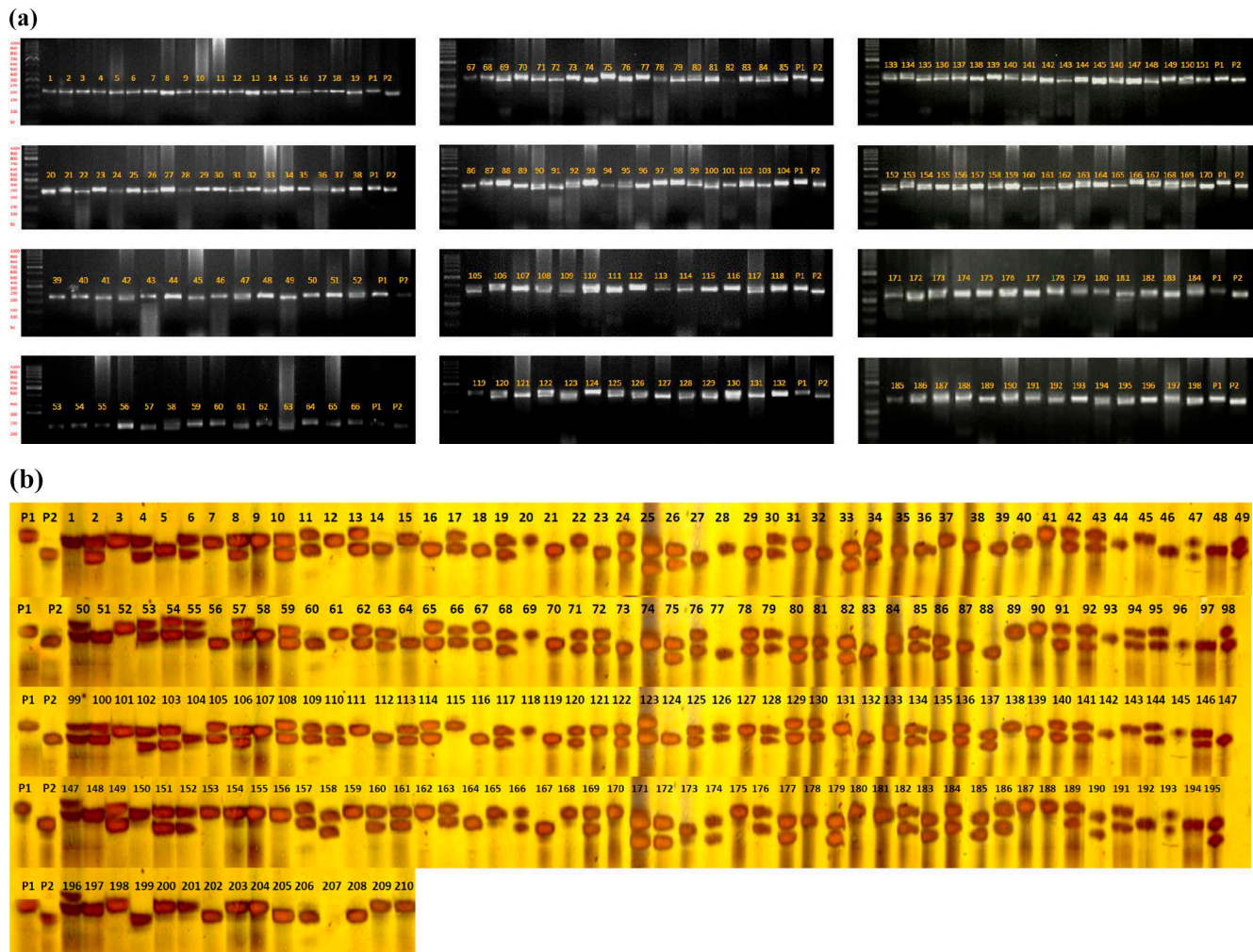


Fig. 4. (a) Segregation pattern of SSR marker GPMS100 on 4% agarose gel in F_2 mapping population derived from the cross *C. annuum* ‘FL 201’ \times *C. galapagoense* ‘TC 07245’. (b) Segregation pattern of AVRDC*MD782 marker on 6% PAGE in F_2 mapping population derived from the cross *C. annuum*. ‘FL 201’ \times *C. galapagoense* ‘TC 07245’.

the genotype TC 07245/TC 07245 had mean value of 5.58, 5.91, 6.04 and 5.58; FL 201/TC 07245 genotype had mean value of 7.53, 8.02, 7.62, 7.89 and marker genotype FL 201/FL 201 had mean value of 8.90, 10.13, 11.47, 10.59 respectively in the F_2 population. These observations suggested that the contributing allele from the *Capsicum annuum* ‘FL 201’ had positive additive effect on fruit length both in homozygous and heterozygous state.

Discussion

An important development during the last few decades has been the ability of crop breeders to tag genomic regions called QTLs responsible for variation in quantitative traits. The linked markers have facilitated genetic enhancement in major field crops through pyramiding of QTLs and breaking of undesirable linkages (Ordon *et al.* 1998, Ribaut and Hoisington 1998). However, the progress in molecular breeding in chilli has been rather slow, especially with re-

spect to the quantitative traits (Barchi *et al.* 2009). This work was undertaken to identify fruit length determining QTLs so as to facilitate their introgression in elite chilli breeding lines through MAB.

The frequency distribution for fruit length in the F_2 mapping population showed nearly normal distribution, which indicated that the trait is quantitatively inherited with additive effects. However, some transgressive segregants on either side of the parental means were observed. This suggested that some positive alleles were also contributed by the short fruited parent ‘TC 07245’. Utilizing such alleles from the wild germplasm, Tanksley *et al.* (1996) increased fruit size in cultivated tomato lines by the introduction of genes from the small fruited *S. pimpinellifolium*. Similarly, red fruit color of a processing tomato line was enhanced by the introgressions from the green fruited *S. hirsutum* (Tanksley and McCouch 1997). The positive alleles detected in ‘TC 07245’ might be of potential use to the chilli breeders. Therefore, it is imperative to identify such genes in ‘TC

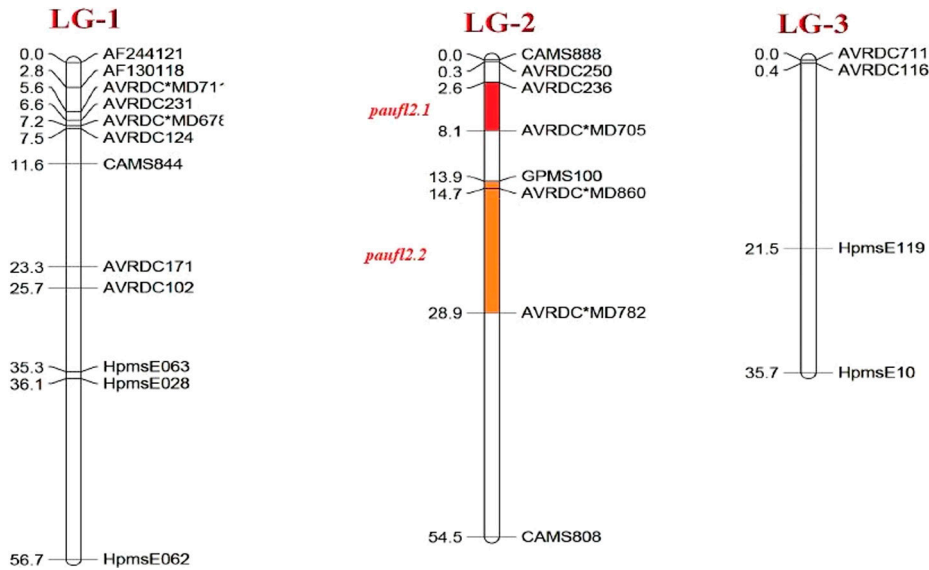


Fig. 5. Genetic linkage map derived from the cross *C. annuum*. ‘FL 201’ × *C. galapagoense* ‘TC 07245’ using Kosambi mapping function. Marker names and the map distances (cM) are indicated on the right and left of linkage groups, respectively. Red colored regions in the linkage map denotes the two fruit length related QTLs designated as *pauf12.1* and *pauf12.2*.

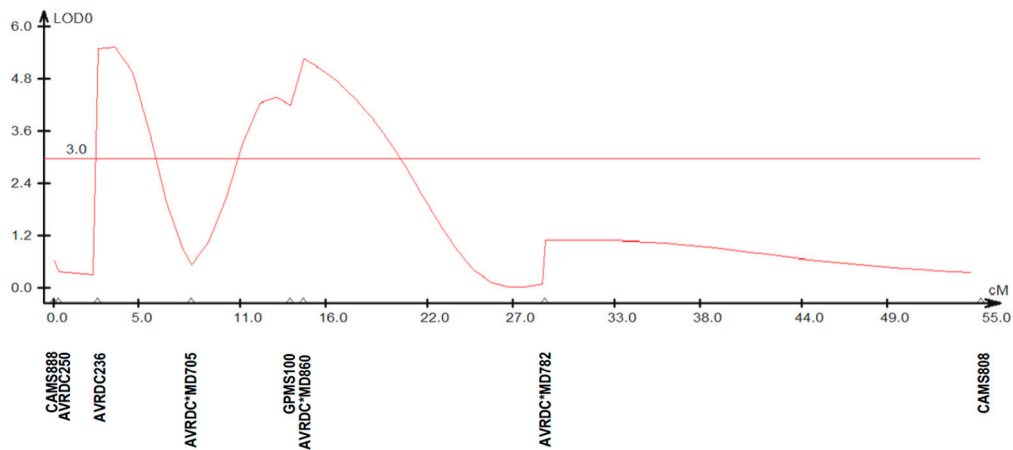


Fig. 6. QTL likelihood plots of LG-2 showing LOD peaks for fruit length in F₂ population. Two peaks depict two fruit length related QTLs designated as *pauf12.1* and *pauf12.2* with LOD peak value of 5.53 and 5.26, respectively. The two QTLs together explained 21.8% of the phenotypic variation.

07245’ and map those on to the *Capsicum* genome.

Our results are consistent with the previous inheritance studies, in that additive gene effects for fruit length are of primary importance (Barchi *et al.* 2009, Ben Chaim *et al.* 2001, Dwivedi *et al.* 2013, Lee *et al.* 2011, Mimura *et al.* 2012) with moderate estimates (0.6–0.8) of h^2_{bs} (Ben Chaim *et al.* 2001, Santos *et al.* 2014). We found 13.7 % polymorphism in our mapping population. The rate of polymorphism is less than the earlier reported by Lee *et al.* (2011), Minamiyama *et al.* (2006) and Moulin *et al.* (2015) but is comparable with those reported by Dhaliwal *et al.* (2014) and Dwivedi *et al.* (2013). Considering that we used high-end resolution MultiNAMicro-chip electrophoresis technique, there are least chances of detecting further polymorphism between the two parents.

Though the parental species were phenotypically diverse, yet the low level of polymorphism could be attributed to their genetic relatedness as both the species have been grouped under Annuum complex (McLeod *et al.* 1983). Secondly, genic SSR markers have been reported to be less polymorphic compared with genomic SSR markers (Cho *et al.* 2000). However, the low level of SSR polymorphism may be compensated for by their potential of inter-specific transferability (Thiel *et al.* 2003).

Most of the QTLs identified by Ben Chaim *et al.* (2001) for fruit and growth characteristics had relatively small effects, controlling less than 20% of the total phenotypic variation, and were clustered in a few chromosomal regions. We identified two fruit length determining QTLs designated as *pauf12.1* and *pauf12.2* clustered together on LG 2. These

QTL regions were not reported in the previous studies by Ben Chaim *et al.* (2001), Dwivedi *et al.* (2013), Han *et al.* (2016) and Rao *et al.* (2003). The two QTLs together contributed 21.78% to the phenotypic variation. QTLs for fruit traits were previously detected in colinear positions on LG 2, LG 3, LG 4 and LG 10 in intra- as well as inter-specific crosses (Barchi *et al.* 2009, Ben Chaim *et al.* 2001, Zygier *et al.* 2005). It is likely that additional QTLs that control this trait might exist that could not be detected in the current study due to low level of parental polymorphisms.

In *Capsicum*, few mapping studies resulting from inter-specific crosses have been conducted. Inter-specific linkage maps developed have originated from the crosses involving *C. annuum* × *C. frutescens* (Ben Chaim *et al.* 2006, Blum *et al.* 2003, Rao *et al.* 2003, Wu *et al.* 2009); *C. annuum* × *C. chinense* (Kang *et al.* 2001, Lee *et al.* 2004, 2009, 2011, Tanksley *et al.* 1988) and *C. annuum* × *C. baccatum* (Eggink *et al.* 2014, Moulin *et al.* 2015). Ours is the first report of the inter-specific partial genetic map developed from the cross *C. annuum* × *C. galapagoense*. The linkage analysis mapped 12 markers on to LG 1, 8 markers on to LG 2, and 4 markers on to LG 3. The genetic map with 24 linked SSR markers containing 3 Linkage groups covers a total distance of 399.0 cM. The genetic map constructed can be considered as one of the precise maps, since it involved the high throughput marker technique and none of the marker showed segregation distortion. The newly mapped markers would enrich the already available *Capsicum* genetic maps and provide wider option to the breeders in selection of the markers, especially with respect to the Linkage groups 1, 2 and 3. However, additional markers are required to generate whole genome linkage map in this cross.

Since SSR markers are transferable between the species, this genetic map will have wider application across the populations. The linked markers will facilitate introgression of the two QTLs identified in *C. annuum* accession 'FL 201' into the diverse genetic background of *C. chinense*, *C. frutescens*, *C. baccatum* and *C. pubescens*. Since fruit length is quantitatively inherited trait with moderate level of heritability, MAB would improve efficiency of selection in the segregating generations. The identified QTLs might also serve as potential target regions for identifying candidate genes through fine mapping. The positive alleles for fruit length present in the short fruited parent 'TC 07245' might be of potential use to the breeders. Therefore, those genes are required to be identified and mapped on to the *Capsicum* genome. Advanced QTL backcross analysis would be useful to simultaneously discover and transfer the positive alleles from the unadapted parent *C. galapagoense* accession 'TC 07245' into elite chilli breeding lines. Secondly, with availability of advanced genotyping techniques, Genomic selection may also help to identify favourable combination of alleles from both the parents based on estimation of genomic assisted breeding values.

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