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# Development of a high resolution <br> intra-specific map of pepper (Capsicum annuum L.), and QTL analysis for horticultural traits. 

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## Introduction

Pepper (Capsicum annuum L.) belongs to the Solanaceae family and it originated from South and Central America where it was domesticated around 7000 BC .

The genus Capsicum includes 25 species, 5 of which are cultivated: Capsicum annuum L., C. frutescens, C. chinense, C. pubescens and C. baccatum (Eshbaugh, 1977). C. chinense originated from the Amazonian region, C. baccatum and C. pubescens had originated from Peru and Bolivia (Pickersgill, 1969), while C. annuum and C. frutescens probably originated from Mexico and central America.
Pepper was introduced in Europe by Cristoforo Colombo after his first voyage to the New World (1492) and his cultivation quickly spread in the European basin.

### 1.1 Crop description

Peppers have been domesticated for 9,000 years. The cultivation has his centre in tropical, subtropical and Mediterranean regions but it spreads all over the world in the area from southern $55^{\circ}$ to northern $52^{\circ}$ latitude. Of the five domesticated species, $C$. annuum is the most cultivated worldwide. The production of pepper for the use as spice and vegetable reached about twenty-three billion tons in 2004 (FAOSTAT 2004, F.A.O., http://faostat.fao.org) with China, Mexico and Turkey as the largest producers. The total number of cultivars at present registered to the official European Union catalogue (G.N.I.S. 2001) reached the number of 749 , behind tomato, lettuce and bean for breeding activity. The chemical compositions of Capsicum fruits include vitamins from the $\mathrm{A}, \mathrm{B}, \mathrm{C}, \mathrm{E}$ and K groups. Vitamin C (ascorbic acid) concentrations range from 0.5 g to 3 g per kg of fresh weight, depending on the variety, a value larger than the one of others fruits. Considerable research has focused on antioxidants in Capsicum which have reported to offer protection
against cancer (Hartwell 1971). Capsanthine and capsorubine are unique carotenoids from the pepper fruits (Davies et al. 1970). They are concentrated in the oleoresin and they are used as colorant by food industries.

Pungency can be considered as the most famous pepper trait. It derives from capsaicinoids, found only in the Capsicum genus, which have presumably a defence role. The amount of these substances in pepper fruits ranges from traces in sweet cultivars to a few percentage of dry weight in the Habanero fruits, the hottest pepper cultivar belonging to $C$. chinense.

### 1.2 Pepper: the plant characteristics

### 1.2.1 Plant morphology

Official descriptors for pepper plants and fruits are available with the aim of classifying the high variability present in the Capsicum genus and to help the characterization of pepper genetic resources. (IPGRI 1995). Pepper plants have a straight primary stem with two to more than 20 internodes, resulting from the growth of its primary apex, which terminates in a flower. Two to three secondary stems develop at the top of the primary stem determining an erected habit. At each internode of the secondary stems, one flower develop in a fruit that is a berry with colors ranging from dark green to ivory-white when immature, and from ivory-yellow to red when mature. Fruit shape ranges from round to ovate or highly elongated with a fruit weight comprised from less than one gram in wild plants to 500 grams for the most productive cultivars (Figure 1). Mature seeds are attached to placenta veins ant their amount is about 120-150 seeds per gram.


Figure. 1 Phenotyphic variation in fruits of different Capsicum species.
Sample of the INRA-Avignon pepper germplasm (picture from Alain Palloix).

### 1.2.2 Reproductive System

Pepper is considered an autogamous plant. However flowers produce a lot of nectar as resulting in a partially allogamous reproductive behaviour, depending on the activity of pollinator insects and the climate. Thus Capsicum can be considered facultative cross -pollinating species (Odland and Porter 1941, Franceschetti 1971, Tanksley 1984). The amount of cross-pollination is a key element in breeding and production. For example in protected cropping system, honeybees are used for the pollination to obtain well shaped fruits with more seeds.

### 1.2.3 Agroclimatic requirements

Several sources of information concerning pepper cultural practices are available (De Witt and Bosland 1993, Nuez et al. 1996, Siviero and Gallerani 1992, Erard 2002). Optimum seeds germination temperature is at $25-30^{\circ} \mathrm{C}$, while the best growing and fruit development temperature is comprised between 21 and $29^{\circ} \mathrm{C}$. (Nonnecke 1989). Temperatures exceeding $32^{\circ} \mathrm{C}$ or lower than $15^{\circ} \mathrm{C}$ lead to a retarding in growth, blossoms falling and yield decreasing (Knott and Deanon 1967). However, flowering and fruit-set return to normality as temperatures return to the optimum (Greenleaf 1986). On the contrary, C. chinense which originated from the Amazonian lowlands, is well adapted to very hot and humid climates. An excess or deficit of water during flowering and fruit set induces flower abortion or further blossom end rot of the fruits. Finally peppers are considered photoperiod-insensitive (Knott and Deanon 1967).

### 1.3 Diseases and pest

Pepper is attacked by several pathogens, including viruses, fungi, oomycetes bacteria and nematodes. Intensive cultivations together with climatic conditions in some cultivation regions favour the development of epidemics.

### 1.3.1 Viral diseases

More than twenty viruses are responsible of attacks on pepper. Viruses which are transmitted in mechanical way (as tobamoviruses) are more frequent in protected crops, while viruses transmitted by insects are prevalent in open fields. Cucumber mosaic virus (CMV) is one of the most important virus diseases of pepper worldwide. CMV is spread by many aphid species in a nonpersistent manner, meaning that insecticides cannot prevent the spread of this disease. Several strains of the virus exist, and all are able to infect pepper with a peculiar symptom expression. The age of a plant at the time of infection strongly influences the severity of epidemics and production losses as well as the type of symptoms (Figure 2). No major genes for CMV resistance have been identified, but several sources of partial resistance have been identified (Pochard and Daubeze 1989, Dufour et al. 1989, Nono-Womdim et al. 1993, Ben Chaim et al. 2001b, Caranta et al. 1997b, Caranta et al. 2002) which conferred a good level of resistance in the field.


Figure 2: CMV symptoms on leaves and fruits (INRA).
Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV) and Pepper mild-mosaic virus (PMMV) are transmitted mechanically by humans and by seeds on the external tegument. Several seed disinfectation methods allow an efficient control. Symptoms include mosaic, necrosis, sterility and malformation and discoloration of fruits (Figure 3). Resistance is conferred by a single dominant gene, the $L$ gene with an allelic series, which control hypersensitive response to the different tobamoviruses.


Figure 3: TMV symptoms on plant and fruits (INRA)

Many potyviruses infect pepper worldwide. Like CMV they are transmitted by aphids in a non persistent manner. Potato virus $Y$ (PVY) is a common virus among Solanaceae, and is prevalent in Europe and Mediterranean countries with strains presenting different pathotypes. Depending on the virus strain-plant genotype interaction, susceptibility results in vein mosaic or necrotic symptoms in leaves and fruits (Figure 4). Tobacco etch virus (TEV), Pepper mottle virus (PepMoV), Pepper severe mosaic virus (PSMV) and Pepper yellow mosaic virus (PYMV) are common in North and South-Central America. Major recessive or dominant resistance genes have been characterized (Kyle and Palloix 1997) and introduced into most modern pepper cultivars. Polygenic and quantitative resistance has also been identified in many local populations from different origins (Kuhn et al. 1989, Caranta and Palloix 1996, Caranta et al. 1997a).


Figure 4: PVY leaf mosaic and vein necrosis (INRA)

Tomato spotted wilt virus (TSWV) can cause disease in a wide variety of plants including pepper, tomato, and lettuce. The virus is common in both temperate and subtropical areas of the world. The virus is transmitted by thrips in a persistent manner with acquisition of the virus by the larvae and transmission after a 4 to 10-day latent period. Symptoms are highly variable, depending on the plant genotype, plant
stage and agroclimatic conditions (Figure 5). In pepper a single dominant gene Tsw confers resistance to TSWV and it has been introduced from C. chinense into C. annuum (Black et al. 1991, Moury et al. 1997). Several commercial hybrids containing the Tsw resistance genes have been commercialised, but new TSWV strains breaking down this resistance gene are known in different production regions.


Figure 5: discoloration of fruits due to TSWV (INRA)

### 1.3.2 Fungi and Oomycetes

Pepper is susceptible to soilborne fungi and Oomycetes both in field and in protected cropping systems. The Oomycete Phytophthora capsici is the major cause of pepper root rot worldwide, causing necrotic lesions on lower stems, branches, leaves and fruits leading to the wilt of the plants and their death (Figure 6). Other important soilborne fungi causing plant wilt are Verticillium dahliae, Phytophthora parasitica, Fusarium oxysporum and Rhizoctonia solani which form a complex in most soils where peppers are repeatedly cultivated.


Figure 6: Collar necrosis and wilt symptoms along irrigation furrows due to Phytophthora capsici; (INRA)

Verticillium dahliae is widespread in temperate climate and it causes plant wilt with an initial dryness of the margin of older leaves. Principal cultural practices that reduce soilborne diseases include drip irrigation and well-drained soils. Soil treatments with chemicals (in particular with the methyl bromide) were applied in greenhouses and allow the reduction of the disease incidence but their utilisation is progressively banned due to their environmental impact. Several sources of partial and polygenic resistance against $P$. capsici and $V$. dahliae were characterized in many local hot pepper cultivars and were introduced in a few large fruited cultivars (Palloix et al. 1990 a and b, Poulos 1994, Thabuis et al. 2004 a and b) but the complex genetic structure of the resistance (Lefebvre and Palloix 1996, Thabuis et al. 2003) makes it difficult to combine a high level of resistance with good agronomic performances

Principal airborne fungi attacking pepper are powdery mildew (Leveillula taurica) which causes plant defoliation, Cercospora capsici causing foliar necrotic ringspots and plant defoliation, and anthracnose caused by Colletotrichum spp which produces mature fruit rot (Figure 7). Several
fungicides may be used against those pathogens however; their efficiency is reduced since the primary infection occurs several weeks before symptoms appearance. Genetic sources of resistance were characterized against powdery mildew, Cercospora capsici and anthracnose in local hot pepper landraces of $C$. annuum and $C$. chinense (Daubèze et al. 1995, Lefebvre et al. 2003, Lim and Kim 2003, Pakdeevaraporn et al. 2004, Voorrips et al 2004).


Figure 7: Defoliation and white sporulations on leaves caused by Leveillula taurica; (INRA)

### 1.3.3 Bacterial diseases

The two main bacterial diseases affecting pepper are bacterial leaf spot, caused by Xanthomonas campestris pv. vesicatoria and bacterial wilt, caused by Ralstonia solanacearum.
$X$. campestris causes necrotic or water-soaked lesions on pepper leaves, fruits and stems. It can survive on leaf surface of many weeds and plant debris (Figure 8) and it is transmitted by contact, rain splashes and seeds. Principal agricultural practices include crop rotation and seed disinfection. Chemicals (in particular copper spray) are useful in pest management but resistant strains were found (Ritchie and Dittapongpitch 1991). Several major genes (Bs1, Bs2, Bs3) were introgressed in pepper cultivars but it was found that they confer a partial
resistance, depending on the prevalent races of the bacteria. A high ability to generate multi-virulent races is noted (Hibberd et al. 1987, Minsavage et al. 1990, Kousik and Ritchie 1998). Alternative sources of resistance were also identified that do not display race (Poulos et al. 1991, Szarka et al. 2002).


Figure 8: leaf necrosis and spots on fruits due to Xanthomonas vesicatoria; (INRA)
R. solanacearum is a soilborne bacterium that infects plant roots, spreads in the plant stems and causes plant wilt at any development stage (Figure 9). R. solanacearum also infects many other hosts including solanaceous crops. Very few preventive measures are efficient, except complex organic soil amendments (Hayward 1991, Fegan and Prior 2005). Partial resistance with oligogenic control to this pathogen was identified in pepper germplasm collections and it is at present available only in local cultivars (Singh and Sood 2003, Kim and Kim 2004, Lafortune et al. 2005).


Figure 9: wilt symptoms and necrosis of vascular tissue of the primary stem due to Ralstonia solanacearum. (INRA)

### 1.3.4 Nematodes

Root-knot nematodes "RKN" (Meloidogyne spp.) are pathogenic on several solanaceous crops, especially peppers, potatoes, and tomatoes (Khan and Haider 1991, Sasser 1977) and are responsible of stunting, wilting or chlorosis (yellowing) with a reduction in plant yield. They are harmful pests particularly under hot climates and under plastic tunnels and greenhouses, where their multiplication occurs continuously (Babaleye 1987). Four species of RKN, Meloidogyne incognita (Kofoid \& White) Chitwood, M. hapla Chitwood, M. javanica (Treub) Chitwood, M. arenaria (Neal) Chitwood cause severe attacks, particularly in the Mediterranean area and in central Africa and America (Fery and Dukes 1984, Lindsey and Clayshulte 1982, Thies et al. 1997). The most common preventive measure was the methyl bromide, but it has been prohibited in industrialized countries since 2005, and will be prohibited in developing countries in 2015 (Federal Register of U.S. Environmental Protection Agency, 2004). New alternatives to methyl bromide have been proposed, as soil solarization which are limited to protected cropping systems and climatic conditions that permit this technique, i.e. soil temperature of $50-55^{\circ} \mathrm{C}$ for at least thirty days. Soil solarization and crop rotation can be combined with chemicals like metham sodium, 1-3-
dichloropropene, or dazomet. The development of new resistant cultivars can be considered as the main alternative to the utilisation of chemicals, particularly in pepper where a wide range of resistance genes with a large spectrum of action were characterized and identified. These included the $N$ dominant gene (efficient only against some Meloidogyne species when transferred into susceptible cultivars), the resistance founded by Di Vito and Saccardo (1979) and Di Vito et al. (1992) in some lines of C. chacoense, C. chinense, and C. frutescens . in addition several dominant genes (Me genes) in C. annuum (DjianCaporalino et al. 2001) were also identified, which display large spectrum and heat stable resistance with different resistance expression ranging from initial limitation of nematode penetration, post-penetration biochemical responses or hypersensitive response (Djian-Caporalino et al. 1999, Pegard et al. 2005).

### 1.4 Cytogenetics

Pepper basic chromosome number is 12, as many other important Solanaceae species (tomato, potato, eggplant). The physical size of the genome (from 2200 to 2700 Mbp ) averages three to four times that of tomato ( 750 Mbp ) or diploid potato ( 1000 Mbp ) (Arumuganathan and Earle 1991). All the Capsicum species present a very homogeneous caryotype with 1 to 2 pairs of acrocentric chromosomes and 11 pairs of meta- or submetacentric chromosomes (Figure10).
The analysis of the inter-specific hybrids considering the five domesticated species indicate exchanges of chromosome ends for seven of the 12. The number and position of translocations correlates with distances between taxa and fertility of hybrids (Gonzalez de Leon 1986, Pickersgill et al. 1991). Isozyme markers were linked with chromosomal interchanges among $C$. annuum, C. chinense and $C$.
baccatum (Tanksley 1984, Gonzales de Leon 1986) while localization of chromosome breakpoints in present maps remain to be established. Information at present available shows that $C$. annuum and $C$. chinense differ by one or two translocations plus one chromosome satellite, affecting chromosome segregation (Lanteri 1991). That may explain the discrepancies between the chromosomes P1 and P8 in the inter-specific map of Livingstone et al. (1999) and the C. annuum intra-specific maps of Lefebvre et al. $(1995,2002)$.


Figure 10: Caryotype of most Capsicum species including most wild $C$. annuum and all C. chinense and C. frutescens (above); caryotype specific to all domesticated and a few wild $C$. annuum (middle); possible variations in the location and number of chromosome satellites ( 1 to 3 ) in the genus (below). (From C. Djian Caporalino et al 2006)

### 1.4.1 Pepper genetic linkage map.

Pochard (1970) selected a complete set of the 12 primary trisomics in the self haploid progeny of $C$. annuum. These 12 were named by using French coding names representing a colour (violet, indigo, bleu, violet, vert, jaune, orange, rouge, pourpre, noir, brun and bistre). Later, 10 monogenic traits and few isozyme markers were mapped in seven of the 12 trisomics (Pochard 1977, Pochard and Dumas de Vaulx 1982, Tanksley 1984). The development of DNA -based markers, in particular with those based on the hybridization analysis of restriction fragment length polymorphism (RFLP) and those based on the polymerase chain reaction (PCR) technique (AFLP, SSR, RAPD SSAP and SNP), allowed the detection of more polymorphism markers than isozyme. Tanksley et al. (1988) and Prince et al. (1993) constructed the first genetic linkage maps of pepper using RFLP markers. Subsequently, both intra-specific and inte-rspecific crosses were used to generate linkage maps (Table 1). Inter-specific maps were generally preferred thanks to their higher level of polymorphism detected (generally up to $80 \%$ ) which allow the genetic map to be more saturated. Parents used for inte-rspecific crosses include C. annum and C. chinense (Livingstone et al. 1999 and Kang et al. 2001), but also C. annuum and C. frutescens (Rao et al. 2003 and Sasvari et al. 2004). C. annuum x C. chinense are generally preferred due to their better compatibility and fertility level. Intra-specific maps were later constructed by Lefebvre et al. $(1995,2002)$, Ben Chaim et al. (2001a), Ogundiwin et al. (2005), and Sugita et al. (2005) which also include maps coming from a cross between a commercial variety and an exotic accession characterized by a high level of resistance to several pathogens. Although inter-specific progenies allow more polymorphisms to be detected than intra-specific progenies, they suffer from segregation distortion, low fertility, and major structural rearrangements (Lanteri 1991; Lanteri and Pickersgill 1993), which limit the power of the linkage analysis and restrict their relevance to markerassisted selection applications. On the contrary, intra-specific maps are
more reliable as they show high fertility level, high recombination rates and undistorted segregation (Lefebvre et al. 2002).

The main limiting aspects in pepper maps are their unsaturated condition and their reduced genome coverage, even if more linkage groups than chromosomes are reported. Thanks to the use of anchor markers (in particular RFLP), the LGs assignment to chromosomes was achieved (Lefevbre et al. 2002), which also allowed the establishment of AFLP homologies among pepper crosses (Lefebvre et al. 2002, Paran et al. 2004).
An integrated map, based on six populations from inter-specific and intra-specific crosses and consisting of 2262 markers covering 1832 cM , has recently been assembled (Paran et al. 2004). This integrated map improved the markers density reaching an average map distance lower than $1 \mathrm{cM}(0.8 \mathrm{cM})$, and also the genome coverage. However, confidence in marker position and order remains low, particularly with regard to population-specific markers which appeared to grouped in terminal parts of chromosome arms. These clusters of population specific markers mainly result from the lack of markers shared between the populations, impeding a stringent integration of the marker position. It also results from chromosomal translocations between genomes from different species. Pickersgill (1991) demonstrated that inter-specific progenies exhibited end-chromosomes exchanges. At least 2 chromosome pairs are concerned by such exchanges between cultivated $C$. annuum and $C$. chinense which were used in mapping.

|  | Parental <br> species | Size <br> and <br> type of <br> progeny | Marker type | No. of <br> marker <br> loci | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Inter- <br> specific <br> maps | C.axC.c | 46 BC | Isozyme and RFLP | 80 | Tanksley et al. 1988 |



Table 1 Molecular maps constructed for pepper genome (adapted from Lefebvre 2004)
These translocations are responsible for errors in mapping and erroneous linkage among different linkage groups as previously reported by Livingstone et al. (1999) and Lefebvre et al (2002) for chromosome P1 and P8. Whatever the mapping population, pepper maps have all
been based on a rather low number of individuals per progeny (commonly between 40 and 100, rarely up to 200), resulting in a poor level of resolution (Lefebvre 2004). As the resolution of a genetic map depends on the number of recombination events in the population, improvement can be achieved by increasing the number of individuals and generations in parallel to the number of markers used to identify recombination events. Mapping in a single large population generates a more reliable and accurate map than an integrated map derived from independent mapping populations.

### 1.4.2 Synteny among pepper and other Solanaceae.

RFLP markers are applicable to several different species allowing the comparison and the alignment of different genomes. Synteny (the preserved linkage of genes between related species) has as purpose to understand species evolution together with genes and noncoding regions function. It also provides a practical interest since basic knowledge from model species can be transferred to syntenic genomes to help locus or gene location. In the Solanaceae family, synteny has been studied using RFLP markers on the four most important species: i.e. pepper, tomato, potato and eggplant. The tomato and pepper chromosomes 2, 4, 6 and 10 are conserved even if some paracentric inversions are present. For the rest of the genome, it seems that only chromosome arms remain conserved together with nonreciprocal or pericentric and paracentric inversions. On the whole, it seems that chromosome breakages occurred at centromeres and certain pepper chromosomes seem to result from more rearrangements of the tomato chromosomes (Tanksley et al. 1988, Livingstone et al. 1999, Lefebvre et al. 2002) (Figure11).


Figure 11: Inter-specific map by Livingstone et al. (1999) and its alignment with the tomato map.
Tanskley et al. (1988) reported that a minimum of 32 breakages of tomato chromosomes must be generated to account for the position of orthologous genes in pepper. This value is certainly underestimated but it is the result of the divergence between the two species.

### 1.5 Mapping traits of interesting

The genetic linkage maps constructed in the last 10 years are powerful tools for mapping major genes and QTLs for both resistance and horticultural traits. Many Mendelian genes and quantitative trait loci controlling qualitative and quantitative resistances to various pests and pathogens have been mapped, as the resistance to Phytophthora capsici, the fungus Leveillula taurica, and the Cucumber mosaic virus (CMV). In addition, genes for resistance to diverse Potyviruses (PVY, TEV, PVMV, and PepMoV), the Tomato spotted wilt virus (TSWV), the root nematodes of the genus Meloigogyne, the tobacco mosaic virus (TMV) and to the bacterium Xanthomonas campestris, have been identified. Moreover several horticultural traits of primary importance for fertility and fruit characteristics were recently mapped (Figure 12).

### 1.5.1 Monogenic and polygenic traits

Several markers are at present available for both horticultural and resistance monogenic traits (Tab.2). The gene cl (chlorophyll retainer) mapped on chromosome P1 have been found to be collinear to the one mapped on tomato chromosome P8 (Efrati et al. 2005) and it could be syntenic to the chromosome P8 in intra-specific maps. Marker linked to C gene was also mapped (Lefebvre et al. 1995, Lefebvre et al. 2002, Blum et al. 2002, Lee et al. 2005, Stewart et al 2005) and it was mapped on chromosome P2. Moreover markers linked to the Me3 and Me4 genes conferring resistance to nematodes were mapped on chromosome P9 (Djian-Caporalino et al. 2001, 2006). With the advent of SSR and SNP markers, new and more reliable markers are today available for the precise identification of monogenic genes of interest, allowing a more precise localization for marker assisted-selection activities.

| Carrierchromosome | Locus name | Effect (gene) | Useful marker | Reference |
| :---: | :---: | :---: | :---: | :---: |
| P1 | cl | Chlorophyll retainer mutation | $\begin{gathered} \hline \text { RFLP (CT28) } \\ \text { at } 3.8 \mathrm{cM} \end{gathered}$ | Efrati et al. 2005 |
| P2 | Bs3 | Resistance to Xanthomonas campestris race 2 | SCAR at 2.1 CM | Pierre et al. 2000 |
| P2? | Bs2* | Resistance to <br> Xanthomonas campestris race 1 (gene encoding a NBSLRR) | AFLP at 0 cM Cloned gene SCAR at 4.9 and 5.3 cM | Tai et al. 1999 a\&b, Kim et al. 2001: <br> Chromosome assignment: unpublished data from INRA Montfavet |
| P2 | C* | Presence/absence of Capsaicinoids (gene encoding acyltransferase 3) | AFLP at 5 cM RFLP (TG205) at 0 cM CAPS at 0.4 cM Cloned gene : 5 SCARs in the gene | Lefebvre et al. 1995, Lefebvre et al. 2002, Blum et al. 2002, Lee et al. 2005, Stewart et al. 2005 |
| P3 | Pvr6 | Resistance to PVMV when associated to $p v r 2^{2}$ gene | $\begin{aligned} & \text { RFLP marker } \\ & \text { (TG057) } \end{aligned}$ | Caranta et al. 1996 |
| P4 | C2* | Orange fruit color (gene encoding phytoene synthase | Cloned gene : RFLP | Thorup et al. 2000, Huh et al. 2001 |
| P4 | Pvr2* | Resistance to PVY(0), PVY(1) (gene encoding elF4E) | Cloned gene : SCAR | Caranta et al. 1997a, Ruffel et al. 2002 |
| P6 | $\mathrm{y}^{*}$ | $\begin{gathered} \text { Red versus yellow } \\ \text { fruit color (gene } \\ \text { encoding } \\ \text { capsanthin- } \\ \text { capsorubin } \\ \text { synthase) } \end{gathered}$ | Cloned gene : SCARs | Lefebvre et al. 1998, Popovsky and Paran 2000 |
| P6 | Rf | Fertility restorer | $\begin{array}{\|c} \hline \text { RAPD at } 0.37 \\ \text { cM } \end{array}$ | Zhang et al.. 2000 |
| P9 | Me3, <br> Me4 | Heat-stable resistance to rootknot nematodes (Meloidogyne spp) | AFLP at 0.5 cM and at 10.0 cM | Djian-Caporalino et al. 2001, 2006 |


| P10 | Pvr4 | Resistance to PVY(0), PVY(1), PVY(1-2) | $\begin{aligned} & \text { CAPS at } 2.1 \\ & \text { cM + SCAR } \end{aligned}$ | Caranta et al. 1999, ArnedoAndres et al. 2002 |
| :---: | :---: | :---: | :---: | :---: |
| P10 | Pvr7 | $\begin{gathered} \text { Resistance to } \\ \text { PVY(0), PVY(1), } \\ P V Y(1-2) \\ \hline \end{gathered}$ | CAPS linked to Pvr4 | Grube et al. 2000a |
| P10 | Tsw | Resistance to TSWV | $\begin{gathered} \text { CAPS at } 0,9 \\ \mathrm{cM} \end{gathered}$ | Moury et al. 2000 |
| P10 | A | Anthocyanin pigments in the tissues | RFLP (TG63) | Chaim et al. 2003 |
| P10 | S* | Soft flesh and deciduous fruit (gene encoding polygalacturonase) | $\begin{aligned} & \text { RFLP at } 0 \mathrm{cM} \\ & (\mathrm{PG}) \end{aligned}$ | Rao and Paran 2003 |
| P11 | L | resistance to TMV | $\begin{gathered} \text { RFLP }(\operatorname{tg} 036) \\ \text { at } 6 \mathrm{cM} \end{gathered}$ | $\begin{gathered} \hline \text { Lefebvre et al. } \\ 1995 \end{gathered}$ |
| P12 | up | Erected fruit | AFLP at 5 cM | Lefebvre et al. $1995$ |
| Unknown | Without name | Stunted growth when associated to the cytoplasm of C. chinense | RAPD at 6 cM | Inai et al. 1993 |

Tab. 2 Major genes mapped on the pepper genome (classified by chromosome number) (Lefebvre 2004, Djian Caporalino et al. 2006).

Moreover these markers can be transferred to different progenies and species and used for LGs assignment and map comparison.

QTL analyses for polygenic resistance to Phytophthora capsici, Leveillula taurica, Potyviruses and Cucumber mosaic virus all revealed the presence of at least one major QTL together with a few lower effect QTLs and epistatic interactions (Lefebvre 2004).

Thanks to RFLP markers, integration of QTLs detected in different studies was possible, allowing the identification of genomic regions (hot spots for resistance) including several R genes and QTIs for resistance to different pathogens (Tab.3). On chromosome P10 a hot spot including
the R genes Pvr4 and Pvr7 for resistance to potyviruses, the Tsw for resistance to TSWV (Grube et al. 2000a), the QTL Pc_10.1 for resistance to Phytophthora capsici and the QTL Lt_10.1 for the resistance to Leveillula taurica was identified (Figure12).

Several studies compared the resistance loci detected in pepper with those detected in tomato and potato. An overall genome comparison was published by Grube et al (2000b) giving evidence for map collocation and synteny of position between the 3 species. Concerning disease resistance, no general rule can be established for functional synteny within the family. For example, the pepper orthologs of tomato Sw and Tm-2 genes already exist in pepper, but resistance to the same pathogens (TSWV and ToMV) is controlled by distinct genes in pepper. Conversely, a few studies delivered expectation for orthology between genes conferring resistance to the same pathogens in the different host species. The Me3 and Me4 locus mapped on pepper chromosome P9 are colinear to the tomato Mi-3 on chromosome T12 and the potato Gpa2 on chromosome XII (Djian-Caporalino et al. 2001). The major resistance pepper QTL to Phytophthora capsici on the chromosome P5 is at a syntenic position with the resistance QTL on tomato chromosome T4 and potato chromosome IV Lefebvre 2004). The two resistance QTLs to powdery mildew due to $L$. taurica mapped on pepper chromosomes P6 and P9 collinear to R genes and QTLs controlling the resistance response to powdery mildew on the tomato chromosomes T6 and T12 (Lefebvre et al. 2003). The tomato pot-1 gene conferring resistance to PVY and TEV is collinear with the pepper pvr2 locus (Parrella et al. 2002) and it was demonstrated to be the same coding gene (Ruffel 2004, Ruffel et al. 2005).
Horticultural traits are more and more investigated (Tab.3). Different genes controlling Mendelian traits, like pungency, fruit colour, fruit texture have been mapped using the candidate gene approach. QTLs controlling horticultural traits were reported by several authors. Ben

Chaim et al. (2001a), using an intra-specific C. annuum cross, analysed a total of 14 horticultural traits, including fruit weight, fruit shape, pedicel diameter but also degree of green and red colour. Rao et al. (2003), using an inter-specific C. annuum x C. frutescens cross, analysed 10 different traits including fruit weight, fruit shape and flowering earliness. Analyses on horticultural traits in pepper were mainly focused on fruit traits, thus no information are at present available on QTLs controlling plant traits other than plant height (Ben Chaim et al. 2001a) and flowering time (Rao et al. 2003). Ben Chiam et al. (2001) revealed a total of 55 QTLs, most of them clustered on chromosomes P2, P3, P4, P8 and P10. In the study performed by Rao et al. (2003), a total of 56 QTLs were identified and the majority of them clustered on chromosomes P1, P2, P3, P4, P8, P10 and P11. These results underline the involvement of these chromosomes in horticultural traits control. Moreover some QTLs detected, in particular for fruit diameter and fruit weight, were closely correlated, as result of the high genetic linkage present among these traits. Several putative orthologous QTLs were identified in pepper. In his work, Rao et al. (2003) stated that 10 QTLs detected could be orthologous with those previously described by Ben Chaim et al. (2001a). These included 3 QTLs for fruit weight (fw2.1, fw3.1 and fw4.1), fl2.1 for fruit length and the major effect QTL on chromosome 3 controlling fruit shape which seems to be conserved in intra-specific crosses and in crosses of C. annuum with C. chinense and C. frutescens (Ben Chaim et al. 2003). Furthermore, several pepper fruit -related QTLs were located in genome regions which correspond to the same traits in tomato (Figure 12). Ben Chaim et al. (2001a) discovered that the QTLs fs3.1, fs8.1 and fs 10.1 controlling the fruit shape in pepper could correspond to tomato fruit shape QTL fs3.1 (Bernacchi et al. 1998), fs8.1 (Grandillo et al. 1996) and fs10.1 (Grandillo et al. 1999; Ku et al. 1999) respectively.

Rao et al (2003) affirmed that only one QTL detected in his progeny and

| Trait | Number of QTLs detected | Effect of the QTLs | Map position of the major QTL | Reference |
| :---: | :---: | :---: | :---: | :---: |
| Number of flowers per node | 2 QTLs | 28.8 + 39.9\% | P2 | Prince et al. 1993 |
| Fruit-related traits | 55 QTLs | 6-67\% according to the QTL | (-) | Ben Chaim et al. 2001a |
| Yield and Fruit related Traits | 58 QTLs | 1-25\% according to the QTL | (-) | $\begin{gathered} \text { Rao et al. } \\ 2003 \end{gathered}$ |
| Fruit shape | 1 QTL fs 3.1 | 2.9-66.7\% according to the trait and the cross | P3 | Ben Chaim et al. 2003 |
| Capsaicinoid content | 1 QTL | 34-38\% according to the experimental | P7 | $\begin{aligned} & \text { Blum et al. } \\ & 2003 \end{aligned}$ |
| Restoration of cytoplasmic male sterility | 5 QTLs | 8-69\% according to the QTL and the trait | P6 | Wang et al. 2004 |
| Resistance to Phytophthora capsici | 7 to 9 QTLs + digenic interactions (depending on the crosses) | 43 to $81 \%$ according to the resistance components | P5 | Lefebvre and Palloix 1996 <br> Thabuis et al. 2003 |
| Resistance to potyviruses | 11 QTLs + 1 digenic interaction | 66 to 76\% according to the potyvirus strain | P4 | Caranta et al. 1997a |
| Restriction of cucumber mosaic virus installation in host-cells | 2 QTLs + 1 <br> digenic interaction | together explaining 57\% of the phenotypic variation | P12 | Caranta et al. 1997b |
| Resistance to cucumber mosaic virus | 4 QTLs + 2 <br> digenic interactions | 7-33\% according to the QTL | P11 | Ben Chaim et al. 2001b |


| Restriction of <br> cucumber <br> mosaic virus <br> long distance <br> movement | 4 QTLs + 2 <br> digenic <br> interactions | $4.0-63.6 \%$ <br> according to the <br> QTL | P 12 | Caranta et <br> al. 2002 |
| :---: | :---: | :---: | :---: | :---: |
| Resistance to <br> Leveillula <br> taurica | 5 QTLs + 2 <br> digenic <br> interactions | together <br> explaining 50\% <br> of the phenotypic <br> variation | $\mathrm{P6}$ and P10 | Lefebvre et <br> al. 2003 |

Tab. 3 Number, effect and map position of QTLs associated with agronomic interest traits in pepper. (Lefebvre 2004, Djian Caporalino et al. 2006).
controlling the fruit shape (fs3.1) could be orthologous to the fs3.1 QTL in tomato. For the fruit weight, Rao et al. (2003) detected 5 QTLs (fw1.1, fw2.1, fw3.1, fw4.1 and fw11.2) which presumably are orthologous to those detected by Grandillo et al. (1999) in tomato. For the same trait, Ben Chaim et al. (2001a) discovered that in their progeny the fw 2.1 and fw3.2 QTLs in pepper could be collinear to fw2.2 in tomato (Alpert and Tanksley 1996) and to fw3.1 in tomato (Grandillo et al. 1999) respectively. Rao et al. (2003) also found that QTLs controlling fruit length (fl2.1 and fl3.1) and fruit diameter (fd1.1, fd2.1, fd4.1 and fd11.2) in pepper could be orthologous in tomato (Lippman and Tanksley 2001). In addition, one pericarp width QTL (perwd1.1) could correspond to a tomato pericarp thickness QTL (Fulton et al. 2000). Finally, the tomato plant height QTL ht3 (deVicente and Tanksley 1993) could correspond to the pepper QTL ph3.1.
Comparisons of QTL position for fruit weight, shape and colour in eggplant with tomato, potato and pepper revealed a partial synteny among Solanaceae (Doganlar et al. 2002). For example the QTL fw2.1 detected in eggplant (Doganlar et al. 2002) seems is colinear to both the major effect fruit weight QTL fw2.2 in tomato (Frary et al.2000) and the QTL fw2.1in pepper (Ben Chaim et al. 2001a). The eggplant QTL ovs4.1
controlling the fruit shape seems to correspond to fs 10.1 in tomato (Grandillo et al. 1999) and pepper (Ben Chaim et al. 2001a).

Others important orthologies have been detected, but they are limited to eggplant, potato and tomato, such as the major anthocyanin QTL in eggplant which seems to correspond to orthologous regions in tomato and potato. In pepper no information are available for many traits already mapped in others species, in particular in tomato, thus a target for future research seems to be the analysis of others traits, with the aim of allowing the comparison of QTLs location in the most important Solanaceae species to better understand the genome organization. Moreover many QTLs detected in pepper seem to be cross specific, and thus not transferable or comparable among pepper crosses and also among species. Furthermore possible orthologous QTLs seem to have different effects. Doganlar et al. (2002) reported that QTL controlling the fruit shape in eggplant ovs4.1 is a are major fruit/ovary shape QTL in eggplant but their putative orthologs in pepper and tomato have relatively minor effects.


Figure12: Genetic map of pepper with localization of major genes and major QTLs for disease resistance and horticultural traits (from Djian-Caporalino et al., 2006). Only the major genes and the major QTLs (those with the highest R2 value for each trait) were positioned. Major QTLs are indicated in rectangular boxes. Abbreviations for QTLs: cap= capsaicin content, $f d=$ fruit diameter, $f /=$ fruit length, $f s=$ fruit shape (ration length/width), $p d=$ pedicel diameter, $p /=$ pedicel length, $c m v=$ resistance to CMV, Phy=resistance to $P$. capsici, Lev=resistance to L. taurica.

### 1.6 Pepper breeding targets

The main breeding objectives in pepper is similar to those for other crop plants and includes the adaptation to abiotic stresses, the improvement in fruit quality and yield, and the resistance to pests and diseases.

### 1.6.1 Adaptation to abiotic stresses

The main abiotic stresses affecting pepper are hydric (including flooding, drought and salinity), energetic (including low light intensity), and supraor suboptimal temperatures which are responsible in yield and quality losses. Abiotic stresses influence traits which are generally quantitative
and polygenic and their interactions have to be taken into account, leading to a hard task for breeders.

### 1.6.2 Horticultural aspects and fruit quality

Breeding for horticultural traits is performed considering standard criteria for every specific cultivar type: i.e. fruit width and length, fruit shape, number of locules and pericarp thickness. These traits are highly variable when considering the large diversity of both local and internationally commercialized cultivars. Large breeding efforts have also been performed concerning plant traits, as plant height, determinate/indeterminate growth, flowering earliness and plant architecture, because those traits determine the plant adaptation to the agro-climatic environment, and to the cultivation system (open field/protected, fresh market/industry).

Breeding for chemical composition is generally focused on high carotenoid content of mature fruit for paprika powder and oleoresin production (food staining). Specific cultivars have been bred with up to 50 times the capsantin/capsorubin content of fresh market cultivars (Hurtado-Hernandez and Smith 1985) Pungency is another key trait governed by the major gene $C$ and by quantitative factors that control the composition and the content in the different capsaicinoids.

### 1.6.3 Disease and Pests

Efforts in breeding for resistance to pests and diseases previously described, firstly tried to introgress resistance components in cultivated varieties. Monogenic resistance to TMV, ToMV and PMMV conferred by the dominant allele at the $L$ locus was introduced in most of the present cultivars, as the resistance to PVY, TEV and nematodes that also depends on major resistance genes. Because virulent variants of these
viral and nematode pathogens were present in the field, new genes or alleles were characterized in genetic resources and progressively introduced in cultivars. No major resistance genes were available against major fungal or oomycete diseases like powdery mildew (due to L. taurica), anthracnose (due to Colletotrichum spp), and Phytophthora root rot, nor against one major virus disease of pepper due to CMV. In those cases, breeders exploited the quantitative resistance components from different accessions. The breeding programs were rather successful with CMV resistance, delivering cultivars with a partial resistance fitting the different cultivation areas. Considering Phytophthora root rot, partially resistant cultivars were also delivered that were successful in North-Mediterranean and temperate cultivation regions. However, the resistance level was proved to be weak in central America, Asia and middle East where the pathogen strains proved to be more aggressive. One of the main target for pepper breeders is now to increase the level of resistance by combining resistance components from non-commercial varieties into productive cultivars.

### 1.7 BREEDING METHODS

Pepper is an autogamous genus and breeders always considered it an inbred. Selection for new cultivars was performed by using massal selection until the 1950's. First breeding activities on scientific basis started by introgressing monogenic resistances to viruses (Greenleaf 1956, Cook and Anderson 1959, Cook 1960) and the creation of high yield cultivars till now used by farmers: i.e Yolo Wonder' and 'Early Calwonder'. The exploitation of F1 hybrids started in 1973 with the release of the 'Lamuyo' cultivar produced by INRA (France).

The use of double haploids lines obtained via polyembryony and in vitro androgenesis proved to be particular useful in reducing time for genetic
analysis of new traits and homozygous line fixation for homogeneous cultivar or FI parental lines selection. The technique was firstly developed by Sibi et al. (1979), Dumas de Vaulx and Chambonnet (1980), and Dumas de Vaulx et al. (1981) and was further improved by several authors (Mityko et al. 1995) with the aim of increasing the rate of haploid regeneration. Up to now, double haploids are used to create new genitors from heterozygous plants which are interesting for certain traits in early stages of pedigree selection (Pochard et al. 1986) and to evaluate genetic gain during selection (Thabuis et al. 2004 a).
Backcrossing programs remain probably the most frequently breeding technique used for introducing major genes of interest coming from exotic accessions into cultivars. The main limit remains the time needed to recover the genomic background of the receiving parent in which genes have been introgressed. Backcrosses also proved ineffective to breed for polygenic resistance due to the progressive loss of favourable alleles. The phenotypic and molecular evaluation of a breeding program was performed by Thabuis et al (2004a), showing that direct backcrossing lead to a decrease of resistance to $P$. capsici associated with the loss of resistance QTLs, whereas intercrossing between resistant populations allowed to increase the number and frequency of favourable alleles. Thus, recurrent selection of multiallelic populations was largely used in pepper for resistance to bacteria (Poulos et al 1991), soilborne Fungi and oomycetes (Palloix et al 1990a, 1990b) and viruses (Palloix et al 1997, Ahmed et al 2001). In these breeding programs, alleles from different genetic origins were progressively associated in elite genotypes, delivering transgressive phenotypes ie. homozygous individuals with higher resistance level than the original parental lines.

### 1.7.1 Marker assisted selection

Marker-assisted selection (MAS) is often considered as the new key technique in breeding program, permitting to increase efficiency in plant breeding compared to phenotypic selection. Thanks to high resolution mapping, markers tightly linked to QTLs of interest for horticultural and resistance traits can indeed improve the selection of favourable alleles at targeted loci. The performance also depends on the capacity to generate specific markers as tools to identify plants carrying genes and QTLs of interest.

The MAS technique has several advantages:
i) The direct selection of the expected allele combination at the QTLs (foreground selection) instead of resulting phenotype.
ii) The reduction of environmental effects on phenotypic evaluation which allows the heritability of the traits to be increase.
iii) The selection of useful genotypes at early stages of development.
iv) The possibility of genetic background selection, accelerating the return to the expected genotype and phenotype when introgressing a new trait (background selection) (Thabuis 2002, Thabuis et al. 2004, Collard et al. 2005)
v) The ability of avoiding the transfer of undesirable or deleterious genes ('linkage drag'; in particular when introgressed genes come from wild species) (Collard et al. 2005).

Limits to MAS generally referred to:
i) The reduce number of loci which can be introgressed. From previous studies (Hospital and Charcosset 1997) the simultaneous introgression of more than 4 QTLs requires large populations or a precise knowledge on the position of QTLs of interest.
ii) The precision of the genetic linkage map used for QTL analysis. A key aspect for the MAS is the availability of markers flanking QTLs of interest at less than 20 cM (confidence interval). More markers are needed when the confidence interval is $>20 \mathrm{cM}$. This leads to the necessity of
developing high resolution genetic linkage maps with the aim of increasing precision in QTL localisation. These maps are based on large number of individuals, which allow to exploit more meiosis, thus increasing the precision in mapping. The number of individuals is also the parameter which more influences the QTL analysis (Moreau et al. 1998): more individuals used for phenotypic tests permit to better dissect the genetic variability of complex traits, thus increasing the precision of QTL detection.
iii) The cost for MAS compared to conventional plant breeding depends by several parameters, i.e. genotyping for map construction (in particular for developing molecular markers), inheritance of the trait, method of phenotypic evaluation, field/glasshouse and labour costs, and the cost of resources. Dreher et al. (2003) indicated that the cost-effectiveness needs to be considered on a case by case basis.
iiii) Efficiency of return to recipient parent. Hospital and Charcosset (1997) stated that after three cycles of backcross the gain in return to recipient parent is limited only to carrier chromosomes. Moreover more cycles could also lead to the loss of QTL donor alleles. Thabuis et al. (2003) showed that after 3 cycles of back cross for introgressing resistance QTLs to Phytophtora capsici, non donor segment remained on the non-carrier chromosomes. In addition, a limited return to recipient parent was observed for carrier chromosomes, which was mainly due to the limit number of plants used for the foreground selection step. Moreover a better knowledge in genome organization is required as unfavourable linkages among traits and recombination between markers and QTLs could be present, which are responsible of the limited effective return to the genome of recipient parent.
v) Limits linked to the inbreed populations which generally show a low level of molecular markers polymorphism. To better apply the MAS strategy, QTLs analysis among different populations which derive from different crosses should be used to detect favourable alleles from
different parents. Thus a population will be created with the target of including the totality of favourable alleles (Ribaut and Betran, 1999).

### 1.8 Objectives of thesis

Up to know, pepper molecular genetic linkage maps have all been based on a rather low number of individuals per progeny (commonly between 40 and 100, rarely up to 200), resulting in a poor level of resolution (Lefebvre 2004). As the resolution of a genetic map depends on the number of recombination events in the population, improvement can be achieved by increasing either the number of individuals and generations and/or the number of markers used to identify recombination events. Mapping in a single population generates a more reliable and accurate map than an integrated map derived from independent mapping populations.

CONCERNING THE HORTICULTURAL TRAITS, FEW INFORMATION ARE AVAILABLE FOR QTL POSITIONS EXCEPT THOSE LOCALISED BY BEN CHAIM ET AL. (2001A) AND RAO ET AL. (2003).(BEN CHAIM ET AL. 2001A). MOREOVER NO INFORMATION ARE AVAILABLE CONCERNING THE RELATIONSHIP BETWEEN RESISTANCE AND HORTICULTURAL QTLS.
Finally another important aspect in QTL analysis is the cost for genotyping and for performing many phenotypic tests with large populations. The chose of a subset of most informative individuals can be a solution, in order to reduce future genotyping and phenotyping effort.

In summary, the aims of the thesis are:

- To develop a new set of SSR markers from genic Capsicum spp. DNA sequence, extending the set reported by Nagy et al. (2004), and describe the potential utility of these assays for the development of high-resolution integrated genetic maps in pepper
- To construct a high resolution genetic linkage map in pepper.
- To perform fine mapping of QTLs linked to horticultural traits using the new genetic linkage map, and confirm the position of QTLs detected by comparing QTLs analysis performed on two different populations.
- To evaluate the effects of the reduction of the size of the population on mapping, trait distribution and QTL detection.
- To examine by different genetic linkage maps, macro-synteny among Solanaceae species.
- To integrate information of genetic locus for resistances and horticultural traits.
- To propose new strategies for Marker Assisted Selection.

Chapter 1: SSR EST-derived development for mapping and phylogenetic studies

Développement de marqueurs SSR dérives de EST pour la cartographie génétique et les études phylogénétiques.


#### Abstract

Various classes of molecular marker have been applied to conduct phylogenetic and mapping studies in pepper, among which microsatellites (SSRs) have been shown to be particularly valuable because of their co-dominant and multi-allelic nature. At present only some of the ca. 180 pepper SSRs available in the public domain have been either inter- and/or intraspecifically mapped. Here we report the development of a set of new ESTbased SSR assays. This class of SSR is more transferable between species than those based on genomic sequence, and so can provide anchor markers in cross-species comparative mapping. A search of $>8,000$ Capsicum ESTs, corresponding to approximately 4.1 Mb of DNA, yielded 1,325 SSRs present in 899 sequences. A cluster analysis was employed to obtain nonredundant SSR-containing sequences, leading to the definition of 576 non-redundant sequences containing 783 SSRs. Primer pairs were designed to amplify 348 SSRs, and a subset of 204 was used to screen for polymorphism within a parental set of four annuum inbred lines and one frutescens accession, used as parents of three mapping populations. The 219 alleles identified at the resulting 49 polymorphic SSRs provided the basis for assessing genetic diversity within an extended genotype set, comprising six annuum and ten wild Capsicum spp. accessions. In this germplasm set, the polymorphism information content per EST-SSR locus lay between 0.24 and 0.86 .


## Introduction

A range of molecular marker types has been applied to study phylogeny and to construct genetic maps in pepper, and of these, microsatellites (or simple sequence repeats, SSRs) are particularly valuable because of their co-dominant, multi-allelic nature (Gupta et al. 1996) and their ready adaptation to automated separation and detection platforms (Nicot et al. 2004). SSRs are abundant and hypervariable in all genomes examined to date (Tautz and Renz 1984; Tautz 1989; Powell et al. 1996; Toth et al. 2000, Kumpatla and Mukhopadhyay 2005). At present about 180 pepper SSRs are available in the public domain, but only some of these have been either inter- or intraspecifically mapped (Lee et al. 2004; Barchi et al. 2006; Minamiyama et al. 2006). However, none were included in a recently published integrated 2,262 locus genetic map (Paran et al. 2004), derived from the analysis of six distinct populations. The further development of pepper SSRs will therefore be valuable as a tool to facilitate mapping in this species.

Conventionally, the development of SSR markers requires the screening of genomic libraries, which may or may not have been previously enriched with SSR-containing fragments (Zane et al. 2002). In recent years, alternative strategies have been developed, in particular by combining the concept of AFLP and the microsatellite-anchor primer technique (Hayden and Sharp 2001; Yang et al. 2001; Hayden et al. 2002; Albertini et al. 2003; Acquadro et al. 2005a, 2005b). However, the increasing public availability of genic DNA sequences provides an substitute route for SSR marker development (Varshney et al. 2005), which was pioneered in the model plant Arabidopsis thaliana (Cardle et al. 2000) and subsequently extended to a range of crop species including barley, maize, durum wheat, rye, sugarcane and grape (Chin 1996; Scott et al. 2000; Temnykh et al. 2000; Cordeiro et al. 2001;

Eujayl et al. 2002; Hackauf and Wehling 2002; Sharopova et al. 2002; Saha et al. 2003; Thiel et al. 2003; Qureshi et al. 2004). By applying this approach to a collection of some 23,000 Capsicum sequences, Nagy et al. (2004) succeeded in developing a set of 50 polymorphic SSRs.

It has been suggested that the frequency of SSRs is inversely related to genome size and to the proportion of repetitive DNA, but its frequency in the gene space (and particularly in the untranslated regions) is independent of genome size (Morgante et al. 2002). From an analysis of more than 1.5 million EST sequences derived from 55 dicotyledonous species, Kumpatla and Mukhopadhyay (2005) estimated that $2.6 \%-16.8 \%$ of ESTs contain at least one SSR. EST-derived SSRs are typically less informative than those from untranscribed regions, but offer a number of advantages over conventional genomic SSRs (Gupta et al. 2003): (i) they detect variation in the expressed portion of the genome, so have the potential to represent "perfect" markers; (ii) their development requires no investment in de novo sequencing; and (iii) the high conservation of primer sites make them readily transferable across species. The latter increases their value to breeding programmes, given their utility as anchors in comparative mapping studies (Varshney et al. 2005).

In this chapter is reported on the development of a new set of SSR markers from genic Capsicum spp. DNA sequence, extending the set reported by Nagy et al. (2004), and the potential utility of these assays for the development of high-resolution integrated genetic maps in pepper is

## Material and methods

## In silico detection of SSR-containing sequences

Since the sequence survey conducted by Nagy et al. (2004), 8,094 new Capsicum sequences (equivalent to some 4.1 Mbp ) have been added to the EMBL nucleotide database (http://www.ebi.ac.uk/emb), over 98\% of these being ESTs. Sequences were retrieved by the Sequence Retrieval System (http://srs6.ebi.ac.uk) and a stand-alone nucleotide database was built allowing local BLAST2 searches (Altschul et al. 1997). First, polyA and polyT tracts were removed, leaving no $(\mathrm{T})_{10}$ or (A)10 in any 10bp window at either end of the sequences. SSR-containing sequences were identified using MISA software, a Perl script (Thiel et al. 2003) which allows both perfect and compound SSRs to be detected. We considered as an SSR any sequence where a repeat motif of one to six nucleotides in length was repeated at least ten times (mononucleotide motif), six times (dinucleotides) or five times (higher-order motifs). For compound repeats, the maximum default interruption (spacer) length was set at 100bp. Redundant sequences were filtered by BLAST analysis, using each individual sequence as a query against the total set of selected sequences. Homologous sequences were aligned using ClustalW (Thompson et al. 1994) and scanned manually in the sequence editor window of the GDE (Genetic Data Environment, version 2.2) package. The criteria for redundancy were (i) where a cluster contained two or more identical sequences, the longest was retained; (ii) where the members of a cluster fell into recognisable sub-groups, one member of each sub-group was retained; (iii) sequences which were composed entirely of SSR motif, lacking any flanking sequence, were discarded since their uniqueness cannot be established and in any event, primer design is not possible. The filtered set were again
subjected to BLAST against all available Capsicum SSR-containing sequences (Huang et al. 2001; Lee et al. 2004; Nagy et al. 2004) to remove already identified loci, and finally, sequences of insufficient length or with poorly defined flanking sequence were discarded. Local BLAST analyses were carried out using all selected SSR markers as queries. The target database contained all 374,630 Solanaceae sequences present in dbEST (http://www.ncbi.nlm.nih.gov/dbEST/) and EMBL databases. The same query sequences were also compared with the 9,554 pepper unigene set available at http://www.sgn.cornell.edu.

## Primer design

Primer pairs were designed from the flanking sequences, using PRIMER3 (Rozen and Skaletzky 2000) in batch mode via the p3_in.pl and p3_out.pl Perl5 scripts within the MISA package. The target amplicon size was set to 100 - 300bp, the optimal annealing temperature to $60^{\circ} \mathrm{C}$, and the optimal primer length to 20bp. Where amplification was unsuccessful, alternative primers were designed by manual input into PRIMER3, using a relaxation of the default parameters. Newly developed SSR markers were given the prefix EPMS (EST Pepper MicroSatellites) attached to a number starting from 595.

## Plant material

The PCR amplification of EST-SSR products, and the initial polymorphism screen were carried out using a set of DNA templates extracted from four C. annuum ('P4', 'GF109', 'YW' and 'CM334') and one C. frutescens ('Tabasco') accession. These genotypes were chosen because they have been used as parents in our three mapping populations ('Tabasco' x 'P4', 'GF109' x 'CM334' and 'YW' x 'CM334').

SSRs displaying polymorphism were subsequently tested against a second DNA panel, containing two annuum and nine wild Capsicum spp. genotypes (Table 1). Genomic DNA was isolated from young leaves following Dellaporta et al. (1983).

## PCR conditions and the detection of SSR amplicons

Amplifications were carried out in $25 \mu$ reactions containing 10-50ng template DNA, 10 mM Tris- $\mathrm{HCl}(\mathrm{pH} 8.3), 50 \mathrm{mM} \mathrm{KCl}, 2.5 \mathrm{mM} \mathrm{MgCl} 2$, 0.5U Taq polymerase, 160 nM labelled forward primer, 200nM unlabeled reverse primer and 0.2 mM dNTPs, using a cycling profile of 1 x $94^{\circ} \mathrm{C} / 3 \mathrm{~min}, \quad 35 \mathrm{x} 92^{\circ} \mathrm{C} / 1 \mathrm{~min}, \quad 60^{\circ} \mathrm{C} / 1 \mathrm{~min}$ and $72^{\circ} \mathrm{C} / 1 \mathrm{~min}$, and 1 x $72^{\circ} \mathrm{C} / 7 \mathrm{~min}$. The forward primers were $5^{\prime}$-labelled with FAM and HEX to allow for amplicon detection after separation on an ABI3100 capillary DNA sequencer (Applied Biosystem Inc., Foster City, CA, USA). Internal size standards were included in each capillary. Fragment data were converted to pseudogel images for analysis by Genographer software (Benham et al. 2001).

## Data analysis

A marker's PIC (polymorphism information content) value reflects the number of alleles and their distribution within a population (Botstein et al. 1980). For our purposes, it was estimated using the equation given by Anderson et al. (1993):
$\mathrm{PIC}=1-\sum_{j=1}^{n} P i j 2$
where Pij is the frequency of the ith allele for marker $i$ and the summation extends over $n$ alleles, calculated for each SSR locus.

The EST-SSR products from the 16 test genotypes were scored as band presence (1) and absence ( 0 ), thus generating a binary matrix. The binary data matrix was used to compute pair-wise similarity coefficients (Nei and Li 1979), and the similarity matrices obtained were utilized to construct a UPGMA-based dendrogram (Sneath and Sokal 1973). The analyses were performed within the software package NTSYS-PC version 2.10 (Rohlf 2002).

## Results and discussion

## SSR frequency and distribution

The in silico analysis identified 1,325 SSRs in 899 (11\%) of the 8,094 Capsicum sequences. Two or more SSR motifs were present in 298 (33\%) of these 899 sequences, including 164 cases of compound SSRs, according to the criteria applied (Table 2). This SSR density (one per 3.1 Kbp ) is about double that estimated across the cereals (barley, maize, rice, rye, sorghum and wheat - Varshney et al. 2002). Inter-SSR distances are typically highly species-specific, ranging from 3.4 Kbp in rice to 11.1 Kbp in tomato (Cardle et al. 2000). The efficiency of SSR identification depends on the criteria applied, and the type of SSR motif. Our higher success rate of SSR identification probably reflects the less stringent than normal length criterion adopted. Furthermore, as di-, triand tetra-nucleotide motifs are those most commonly targeted for marker development, most analyses to date have ignored mononucleotide motifs (Kantety et al. 2002; Eujayl et al. 2004; Saha et al. 2004; Park et al. 2005; Chen et al. 2006; Han et al. 2006). However, we have included this class, as they have proven useful for filling gaps in
linkage maps constructed from di-, tri and tetra-nucleotide SSRs (Kumpatla and Mukhopadhyay 2005).

Seven large sequences (the 104Kbp YAC clone AY702979, and six genomic or full-length cDNA sequences representing known genes Table 2) were removed from the sequence set subjected to automated sequence analysis. To avoid an overestimation of specific SSR types, a redundancy analysis was performed on the 899 SSR-containing sequences, which resolved these into 99 clusters containing 422 sequences, and 477 singletons. The retention of only one member from each cluster produced a non-redundant set of 576 sequences $(7.1 \%$ of the sequences investigated, Table 2), containing 783 SSRs. Of these, 531 (68\%) were mononucleotide motifs, 103 (13\%) dinucleotide, 137 (18\%) trinucleotide, 8 (1\%) tetranucleotide, 1 penta- and 3 hexanucleotide (Table 2). The high abundance of mononucleotide SSRs is consistent with prior analyses of SSR presence in plant genomes (Wang et al. 1994; Cardle et al. 2000; Morgante et al. 2002). Trimeric repeats are more common than dimeric ones in the gene-space of both human (Subramanian et al. 2003) and a number of plant species (Cardle et al. 2000; Scott et al. 2000; Temnykh et al. 2000; Morgante et al. 2002; Varshney et al. 2002; Thiel et al. 2003). It has been hypothesised that this feature has evolved as a result of the suppression of non-trimeric SSRs, as variation in the repeat number of these SSRs will frequently result in frameshift mutations (Metzgar et al. 2000) - unlike the situation for trinucleotide SSRs, where repeat unit variation does not normally disturb frameshift.

The occurrence of the various SSR motifs is summarized in Table 3. The frequency of a given SSR type is of interest not only to allow a better understanding of genome structure to be gained, but also provides a guide to increase the efficiency of SSR development (Kumpatla and Mukhopadhyay 2005). Among the dimeric SSRs, the most abundant motif was GA/CT (58\%), similar to the situation
pertaining in a wide array of species (Temnykh et al. 2000; Kantety et al. 2002; Thiel et al. 2003; Fraser et al. 2004; Nicot et al. 2004; Saha et al. 2004; Kumpatla and Mukhopadhyay 2005). The CG motif was absent. Dinucleotide motifs are present in a number of codons - for example, GA/CT occurs in (GAG) $)_{n}$, (AGA) $)_{n}$, (UCU) $)_{n}$ and (CUC) $)_{n}$, translating into polyArg, Glu, Ala and Leu respectively. Ala and Leu are present in polypeptides at frequencies of $8 \%$ and $10 \%$, respectively (Lewin, 1994), providing one of the major reasons why GA/CT motifs are present at such high frequencies in EST collections (Kantety et al. 2002). AT SSRs are typically abundant in plant genomic sequence (Lagercrantz et al. 1993; Morgante and Olivieri 1993), and they are also well represented among the pepper EST sequences ( $26 \%$ of the dimeric motifs, Table 3); in contrast, they are under-represented in barley (Thiel et al. 2003), rice (Temnykh et al. 2000) and maize (Chin 1996).

AAG/CTT was the most common trimeric motif present in pepper, and AAAT/ATTT the most common tetrameric one (Table 3). AAG repeats are common in both A. thaliana (Cardle et al. 2000) and Gossypium spp. (Park et al. 2005), while CCG predominates in barley, rice, maize, sorghum and sugarcane (Temnykh et al. 2000; Cordeiro et al. 2001; Kantety et al. 2002; Thiel et al. 2003; Nicot et al. 2004), and AAC in wheat (Kantety et al. 2002). Presumably, these differences vary across genomic regions (coding or non-coding) and/or different organisms (Park et al. 2005), but it is difficult to compare the prevalence of a particular motif across different plant species due to the use of mutually inconsistent search and retention criteria by different studies. Table 4 summarises the properties of the 783 SSR loci present in nonredundant sequences, classified on the basis of repeat motif and number of repeat units. Most of trimeric ( $84 \%$ ) and dimeric (65\%) SSRs showed 5-6 or 6-7 repeats, respectively, consistent with the mean size of barley EST-SSRs (Thiel et al. 2003), and with range recorded among the 55 dicotyledonous species by Kumpatla and Mukhopadhyay (2005).

The cluster analysis showed that 44 of the 576 non-redundant SSR-containing sequences were highly similar to those exploited previously (Nagy et al. 2004; Lee et al. 2004), and so 532 sequences (6.6\% of the original sequences) were taken forward for the development of new SSR markers. The seven long sequences (>1.5Kbp) were manually screened for SSR motifs, generating 57 SSRs, 39 of which contained mononucleotide and 12 dinucleotide repeats. Among the latter, AT was the most common motif, and CG repeats were totally absent (Table 3).

## SSR-marker development

Of the 532 SSR containing sequences, 319 (60\%) were suitable for primer design (EPMS-584 to EPMS-902), comprising 158 mononucleotide, 34 dinucleotide, 90 trinucleotide, 5 tetranucleotide, 3 hexanucleotide and 29 compound SSRs (see supplementary material). The remaining sequences contained either too little flanking sequence, or the sequences themselves were refractory for primer design. Thus, overall SSR primers could be designed to amplify non-redundant loci from about 4\% of the initial number of ESTs, a figure comparable to that found in other species. A further 29 primer pairs (EPMS-903 to EPMS931, see supplementary material) were generated from among the 57 SSRs present in six of the seven long sequences.

From the 348 pepper SSRs developed, a subset of 204 primer pairs were finally selected for a polymorphism survey, by applying a minimum repeat length criterion of 18 repeats for $\operatorname{poly}(A)$ and $\operatorname{poly}(T)$ mononucleotides, and seven for all dinucleotides. This subset comprised 28 mononucleotide and 20 dinucleotide while all the higher-order and compound repeats were included.

## Evaluation of marker polymorphism

The initial survey of the five mapping parents showed that dinucleotide motifs, although under-represented, were more informative than the trinucleotide repeats. A low variability of trimeric EST-SSR loci has also been demonstrated in both rice (Cho et al. 2000) and pine (Liewlaksaneeyanawin et al. 2004). The 49 EST-derived SSR-markers useful for genetic mapping are presented in Table 5; 47 of these detect polymorphism between 'Tabasco' and 'P4' and are thus suitable for interspecific mapping, while 21 are mappable in both intra-specific populations. Overall, $49 \%$ of the polymorphic markers were mappable in one population, $18 \%$ in two and $31 \%$ in all three mapping populations. Amplicons of expected size were obtained in $80 \%$ of cases. In five cases, however, larger than expected amplicons were generated, presumably the outcome of interruption by intronic sequence; while in the remaining five cases, a smaller than expected amplicon was generated. This unexpected result has been suggested by Nicot et al. (2004) to be generated by a combination of (1) the occurrence of deletions within the genomic sequence; (2) a lack of priming specificity, resulting in the amplification of a non-target member of a gene family; or (3) the presence of minor sequence variation between the amplified copy and the consensus sequence.

The PIC value of each of the 49 mappable markers was based on the allelic constitution of all 16 templates (Table 1). The number of alleles and the range in allele size are reported in Table 5. Allele number varied from 2 to 9 (mean 4.5). The largest variation in amplicon size (261-349bp) was present at EPMS-923. PIC values ranged from 0.24 to 0.86 (mean $0.62 \pm 0.02$ ). EPMS-650 had the highest PIC, and EPMS603 and EPMS-670 the lowest. The high level of interspecific SSR transferability achieved across the eight Capsicum species included in
the present study mirrors that experienced in other plant contexts (reviewed by Varshney et al. 2002), which have confirmed that ESTSSRs are highly conserved across species (Scott et al. 2000). This conservation may act to inhibit the level of intraspecific polymorphism present - thus it was unsurprising to find that only $24 \%$ of the ESTderived SSRs were informative in the interspecific mapping populations. This level of polymorphism is typical of other species (Eujayl et al. 2002; Han et al. 2004; Park et al. 2005), and compares poorly with the rate achievable from SSR assays derived from sequence extracted from enriched genomic DNA libraries (Reddy et al. 2001; Eujayl et al. 2002; Nguyen et al. 2004).

## Diversity analysis

The usefulness in the context of diversity analyses of the derived ESTSSR markers was investigated by assessing genetic similarity (GS) among the 16 test genotypes. GS varied from 0.09 ('Tabasco' vs. 'CM334') to 0.81.('Chi1' vs. 'Chi7'). Genetic relationships between the accessions was in good agreement with known taxonomic classifications. Thus $C$. annuum types were clustered together, and two sub-clusters corresponding to fruit morphology characteristics could be recognised - the small-fruited types ('Perennial','MN1' and 'CM334') being clearly separated from the large-fruited ones. The cluster closest to the annuum group contained both frutescens and chinense types, consistent with the taxonomic model which groups these three species into a single complex (Pickersgill 1988). The third cluster contained the remaining wild Capsicum species baccatum (vars. baccatum and pendulum), praetermissum, chacoense, eximium and pubescens (Figure 1). Thiel et al. (2003) have stressed some limitations of the application of SSR markers for diversity studies, emphasising the possibility of homoplasy (identical allele sizes may not be identical by descent), and
pointing out that alleles of different size can be generated by indel events, as well as by variation in the number of SSR repeats. However, the SSR marker-based genetic relationships among the Capsicum species reported here are in agreement to those generated by RFLP, RAPD and AFLP markers (Prince et al. 1995; Rodriguez et al. 1999; Portis et al. 2006).

## Conclusion

This study was designed to supplement ongoing efforts to develop and exploit EST-SSRs in pepper (Huang et al. 2001; Lee et al. 2004; Nagy et al. 2004). The newly-derived EST-SSRs are informative for phylogenetic and mapping studies. Since EST-SSRs are present within the transcriptome, these assays may represent a valuable means to map and identify genes of known function related to phenotypic variation.

Table 1 Capsicum accessions assayed for the study of EST-SSR variation.

## Variety/Inbred line/ Accession ${ }^{\text {a }} \quad$ Taxonomy

## Sample panel 1:

Tabasco (Tab) C. frutescens
P4 - red paprika
GF109 - red paprika C. annuum
Yolo Wonder (YW) - green to red C. annuum
blocky
C. annuum

Criollo de Morellos 334 (CM334) -
C. annuum

Chile

## Sample panel 2:

| Perennial (Per) - Chile | C. annuum |
| :--- | :--- |
| MN1 - purple leaf ornamental | C. annuum |
| Fru48 | C. frutescens |
| Chi1 | C. chinense |
| Chi7 | C. chinense |
| Pub3 | C. pubescens |
| Cha6 | C. chacoense |
| Exi1 | C. eximium |
| Bac20 | C. baccatum var. |
| Pen6 | baccatum |
| Pra1 | C. baccatum var. |
|  | pendulum |
|  | C. praetermissum |

${ }^{\text {a }}$ Sample panel 1 was used to test PCR amplification and polymorphisms for 204 primer pairs. The primer pairs that

SSR development - Développement de marqueurs SSR
detected polymorphism in sample panel 1 were tested further using DNAs from both sample panels to calculate PIC values.
SSR development

|  | Total | NR | NA | Sequences > 1.5 <br> Kbp |
| :---: | :---: | :---: | :---: | :---: |
| Total number of sequences examined: | 8094 | 576 | 532 | 7 |
| Number of SSR containing sequences: | 899 | 576 | 532 | 7 |
| Total number of identified SSRs: | 1325 | 783 | 727 | 57 |
| Total size of SSR containing sequences |  |  |  |  |
| (Kbp): | 579.1 | 283.8 | 257.7 | 120.6 |
| Number of sequences containing more |  |  |  |  |
| than 1 SSR: | 298 | 157 | 146 | 4 |
| Number of SSRs present in compound |  |  |  |  |
| formation: | 164 | 112 | 107 | 7 |
| Mono | 936 | 531 | 512 | 39 |
| By | 167 | 103 | 88 | 12 |
| Tri | 207 | 137 | 116 | 4 |
| Tertra | 11 | 8 | 7 | 2 |
| Penta | 1 | 1 | 1 | - |
| Hexa | 3 | 3 | 3 | - |

Table 3 Frequency of repeat motifs. NR: non-redundant SSRcontaining sequences; NA: sequence not aligned with previously developed EST-SSRs.

| SSR motif | Total | NR | NA | Sequence$\begin{gathered} s>1.5 \\ \text { Kbp } \end{gathered}$ |
| :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  |
|  |  |  |  |  |
| A/T | 903 | 506 | 488 | 35 |
| C/G | 33 | 25 | 24 | 4 |
| AC/GT | 21 | 16 | 14 | 1 |
| AG/CT | 99 | 60 | 51 | 2 |
| AT/AT | 47 | 27 | 23 | 9 |
| AAC/GTT | 20 | 19 | 12 | 1 |
| AAG/CTT | 61 | 40 | 36 |  |
| AAT/ATT | 25 | 17 | 14 | 1 |
| ACC/GGT | 35 | 15 | 12 | 2 |
| ACG/CTG | 4 | 3 | 3 | - |
| ACT/ATG | 9 | 9 | 9 | - |
| AGC/CGT | 11 | 11 | 9 | - |
| AGG/CCT | 13 | 7 | 6 | - |
| AGT/ATC | 16 | 10 | 10 | - |
| CCG/CGG | 13 | 6 | 5 | - |
| AAAG/CTTT | 1 | 1 | 1 | - |
| AAAT/ATTT | 4 | 3 | 2 | 1 |
| AAGG/CCTT | 1 | 1 | 1 | - |
| AATT/AATT | 3 | 2 | 2 | - |
| ACAT/ATGT | 2 | 1 | 1 | 1 |
| AAGAG/CTCTT | 1 | 1 | 1 | - |

SSR development

| AAAAAC/GTTTTT | 1 | 1 | 1 | - |
| :--- | :--- | :--- | :--- | :--- |
| AACCCG/CTTGG | 1 | 1 | 1 | - |
| G <br> ACGAGG/CCTGC <br> T | 1 | 1 | 1 | - |
| Total | 1325 | 783 | 727 | 57 |

Table 4 Occurrence of non-redundant SSRs in a set of ca. 8,000 Capsicum sequences.

| SSR motif | 5 | 6 | 7 | 8 | 9 | i. | Number of repeats |  |  |  |  | >15 | Total |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  |  | 10 | 11 | 12 | 13 | 14 | 15 |  |  |
| A/T | - | - | - | - | - | 92 | 50 | 40 | 30 | 20 | 20 | 254 | 506 |
| C/G | - | - | - | - | - | 8 |  | 7 | 3 | 1 | 2 | 4 | 25 |
| AC/GT | - | 9 | 3 |  |  | 2 | 1 | 1 |  |  |  |  | 16 |
| AG/CT | - | 20 | 16 | 7 | 2 | 2 |  | 1 |  |  | 1 | 11 | 60 |
| AT/AT | - | 10 | 9 | 1 | 1 | 2 |  | 1 |  |  | 1 | 2 | 27 |
| AAC/GTT | 11 | 4 | 2 | 1 | 1 |  |  |  |  |  |  |  | 19 |
| AAG/CTT | 25 | 9 | 3 | 1 | 1 | 1 |  |  |  |  |  |  | 40 |
| AAT/ATT | 5 | 5 | 4 | 2 |  |  |  | 1 |  |  |  |  | 17 |
| ACC/GGT | 11 | 2 |  | 1 | 1 |  |  |  |  |  |  |  | 15 |
| ACG/CTG | 2 | 1 |  |  |  |  |  |  |  |  |  |  | 3 |
| ACT/ATG | 7 | 1 | 1 |  |  |  |  |  |  |  |  |  | 9 |
| AGC/CGT | 7 | 4 |  |  |  |  |  |  |  |  |  |  | 11 |
| AGG/CCT | 5 | 2 |  |  |  |  |  |  |  |  |  |  | 7 |
| AGT/ATC | 9 |  | 1 |  |  |  |  |  |  |  |  |  | 10 |
| CCG/GGG | 4 | 1 | 1 |  |  |  |  |  |  |  |  |  | 6 |
| AAAG/CTTT | 1 |  |  |  |  |  |  |  |  |  |  |  | 1 |
| AAAT/ATTT | 1 | 2 |  |  |  |  |  |  |  |  |  |  | 3 |
| AAGG/CCTT | 1 |  |  |  |  |  |  |  |  |  |  |  | 1 |
| AATT/AATT | 2 |  |  |  |  |  |  |  |  |  |  |  | 2 |
| ACAT/ATGT |  |  | 1 |  |  |  |  |  |  |  |  |  | 1 |
| AAGAG/CTCTT | 1 |  |  |  |  |  |  |  |  |  |  |  | 1 |
| AAAAAC/GTTTTT | 1 |  |  |  |  |  |  |  |  |  |  |  | 1 |
| AACCCG/CTTGGG | 1 |  |  |  |  |  |  |  |  |  |  |  | 1 |
| ACGAGG/CCTGCT | 1 |  |  |  |  |  |  |  |  |  |  |  | 1 |



| Name | Accession ID ${ }^{\text {a }}$ | Description | SSR <br> type | Primer sequences (forward , reverse) | Size <br> (bp) | Parental test ${ }^{b}$ | Product (bp) | $\mathrm{N}^{\circ}$ <br> alleles | PIC | Homol. unigenes |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-596 | CO907458 | C. annuum cDNA | (A) ${ }_{19}$ | CTCGTGCCGTATTTCTGTCA AAGGGCGTGTTTGGTATGAA | 250 | TP | 235-240 | 3 | 0.64 |  |
| EPMS-600 | CO776120 | C. annuum cDNA | (A) ${ }_{32}$ | ATGGGTACGTGTtTGGGGTA ACTTTATTCCTCGTGCCGAA | 210 | TP | 210-218 | 3 | 0.56 |  |
| EPMS-601 | CO776313 | C. annuum cDNA | (A) 34 | AAATTGAGAACATCGGTGCC TAAAGAAAGAGCCTCGTGCC | 230 | TP | 230-235 | 3 | 0.33 | U204884 |
| EPMS-603 | CO907570 | C. annuum cDNA | (A) 39 | GCGGTTCCCTATtTGAAGAA ATAGGGGGAATTGGGTTCC | 164 | TP | 231-234 | 2 | 0.24 | U196266 |
| EPMS-628 | CO907462 | C. annuum cDNA | (T) ${ }_{19}$ | TGCTCCTTAAGACTGGCACC GGGTTCGGCTCTGTTATTGA | 280 | TP | 142-148 | 3 | 0.64 |  |
| EPMS-629 | CO908660 | C. annuum cDNA | (T) ${ }_{19}$ | GCTCGAGGGAGAGAGACTGT GGTCATATGTTTCCATGGGC | 228 | TP-YC | 220-225 | 3 | 0.42 | U203329 |
| EPMS-642 | CO911525 | C. annuum cDNA | $(\mathrm{AT})_{8}$ | CAACTTCGCGTTATTGTCCA AGGGCGGACAAAGAAGATTT | 187 | TP | 180-210 | 5 | 0.71 |  |


|  |  |  |  | SSR development |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-643 | CO908387 | C. annuum cDNA | (CT) $1_{17}$ | CCAAGATCAACTCTTACGCTATC CCCTCAAGAATTCCCTCCAT | 212 | TP-GC-YC | 195-223 | 9 | 0.82 |  |
| EPMS-648 | CO907649 | C. annuum cDNA | $(\mathrm{GA})_{7}$ | TGTAAAATAAAATAAGGCTAAAGGCA CAAGAAAGTGTGCCCCAAAT | 167 | TP-YC | 165-190 | 4 | 0.49 |  |
| EPMS-649 | C0776131 | C. annuum cDNA | (TA) 12 | AAGGGTTCTCGAGGAAATGC TCAATCCCAAAACCATGTGA | 263 | TP-YC | 249-281 | 8 | 0.76 |  |
| EPMS-650 | CO910134 | C. annuum cDNA | (TA) $1_{19}$ | CATGGGTGAGGGTACATGGT AGAGGGAAGGGTTATTTGCC | 251 | TP-GC-YC | 237-261 | 8 | 0.86 | U203201 |
| EPMS-654 | CO776379 | C. annuum cDNA | (AAC) 5 | TTCCACTCTTCGAAGCACCT GGTAGGGTTTAACACCGCCT | 262 | TP | 263-267 | 4 | 0.72 | U203002 |
|  |  | C. annuum |  |  |  |  |  |  |  |  |
| EPMS-657 | CK901687 | Root cDNA clone similar to coldstress inducible protein | (AAG) 5 | CTGATCGTGGATGTGGATTG TAGAATTGCTGTGAGTGCGG | 165 | TP | 159-168 | 4 | 0.59 |  |
| EPMS-658 | CO906736 | C. annuum cDNA | (AAG) <br> 5 | CCTTGAGTAGGCGCACAAAT <br> TTCCTCATTGCTTTTCCCAC | 142 | TP | 139-160 | 6 | 0.58 |  |


| EPMS-669 | AY488030 | C. annuum ly200 protein mRNA | (AGA) ${ }_{5}$ | AGAGCAGGCGAAACCCTAAT GGCATCAAGACACATCCCTT | 214 | TP | 290-296 | 3 | 0.30 | U196446 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-670 | CO910307 | C. annuum cDNA | (AGA) ${ }_{5}$ | TCACAAAGATGGAGAAGGGAA CAATCACTGTCACTGCTACTGCT | 102 | GC-YC | 107-110 | 2 | 0.24 |  |
| EPMS-676 | CO910259 | C. annuum cDNA | (ATA) ${ }_{7}$ | TGGTGAACATGAAGAGCCAA TTTAGCTGTTTGCCATGCAC | 214 | TP | 201-223 | 5 | 0.55 |  |
| EPMS-677 | CO909968 | C. annuum cDNA | (ATA) 8 | ATCTGCCCTTATCGATGCAC CCGAATTGTGGAGGAAACAT | 216 | TP | 200-212 | 4 | 0.68 |  |
| EPMS-680 | CO911150 | C. annuum cDNA | (ATT) ${ }_{6}$ | TGGAATTCACATGGTGAAAAA TGAAACTTTGTGGGCTATGG | 279 | TP | 267-284 | 4 | 0.63 |  |
| EPMS-683 | CO907354 | C. annuum cDNA | (CAA) ${ }_{7}$ | AAATGGATCCCAACAACCAA GGAGTTGAAAACGGTGGAGA | 200 | TP-GC | 184-206 | 5 | 0.71 | U197147 |
| EPMS-686 | CK901658 | C. annuum Root cDNA clone | (CAG) ${ }_{6}$ | CAAAGAAGGAAGGGGGTTTC CTCCCTCTTGTTCCTTGCAC | 158 | TP-YC | 152-158 | 3 | 0.58 |  |
| EPMS-689 | CO910607 | C. annuum cDNA | $(C A T)_{7}$ | AGAGGGGGTACTCATTGGCT TCGGAGGAAGAGGAAGAACA | 272 | $\begin{aligned} & \text { TP-GC- } \\ & \text { YC } \end{aligned}$ | 253-275 | 4 | 0.47 | U200269 |


| SSR development |  |  |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-694 | CO912323 | C. annuum cDNA | $(C C A) 8$ | CTAGTACGAGGCAGGGGAGG CCAGATCCCGCTTTTGACTA | 238 | TP-GC-YC | 228-254 | 6 | 0.74 |  |
| EPMS-697 | CO911728 | C. annuum cDNA | $(C T G) 5$ | ATGTCGCTCGCAATTTCACT CGTAGGGAGGAGCGATAGAG | 120 | TP-GC-YC | 110-120 | 4 | 0.54 |  |
| EPMS-703 | CO776113 | C. annuum cDNA | (GAA) ${ }_{5}$ | AAGATtTGGCGGAGACTTCA TGCACCAACTTTGTCTCTGC | 104 | TP | 106-118 | 6 | 0.69 | U197414 |
| EPMS-704 | CO910889 | C. annuum cDNA | (GAA) ${ }_{5}$ | GGTCCTCTGATTGGCAACAT GACCTGAAATTGGAGCAAACA | 126 | GC-YC | 126-134 | 3 | 0.52 |  |
| EPMS-705 | CO909594 | C. annuum cDNA | (GAA) 5 | TCAACTAGATCCACCACGCA taAcCCGTTGCTCACACTCA | 106 | TP | 105-114 | 3 | 0.65 |  |
| EPMS-709 | CO909799 | C. annuum cDNA | (GAG)6 | ACGCCGAGGACTATGATGAC TTCTTCATCCTCAGCGTGTG | 272 | TP-GC | 262-280 | 7 | 0.79 | U196437 |
| EPMS-712 | CK902011 | C. annuum <br> Root cDNA <br> clone similar to | $(\mathrm{GCA})_{6}$ | CCACAAAGGGTTTAAGCAGC aAGGCAGGAGCAGAGTTCAA | 148 | TP-GC | 140-146 | 3 | 0.54 |  |
|  |  | At1g01160/F6 <br> F3_1 protein |  |  |  |  |  |  |  |  |
| 63 |  |  |  |  |  |  |  |  |  |  |


|  |  | C. annuum |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-715 | CK901642 | Root cDNA clone similar to WRKY transcription factor 41 protein | (GGT) ${ }_{5}$ | GTGCCAAACATCCAAGAGGT ACCAAAGGTAGTGGTGCTGG | 196 | TP | 180-196 | 3 | 0.60 |  |
| EPMS-716 | C0775942 | C. annuum cDNA | $\begin{aligned} & (\mathrm{TAA})_{1} \\ & 2 \end{aligned}$ | CAGGAGATCCGCAGGATAAG tTtTAAAGTTGATCAAGCGTAATTTTT | 223 | TP-GC-YC | 194-232 | 7 | 0.84 |  |
| EPMS-718 | CO912216 | C. annuum cDNA | (TAT) ${ }_{5}$ | TCACTCGTTATATTTTTCTGTCTCG CTGCTGAAGCGTGGTGAATA | 229 | TP | 210-234 | 6 | 0.76 | U202237 |
| EPMS-725 | C0910566 | C. annuum cDNA | $(\mathrm{TCT})_{8}$ | TTGAATCGTTGAAGCCCATT ATCTGAAGCTGGGCTCCTTT | 158 | TP-GC-YC | 142-157 | 5 | 0.73 |  |
| EPMS-745 | CO907754 | C. annuum cDNA | (ATAC <br> $)_{7}$ | GTTGTTGGGTGGTACTTGGG GGAAGATCTCAAATGGGTCG | 223 | TP-GC-YC | 212-231 | 4 | 0.69 |  |
| EPMS-747 | C0907904 | C. annuum cDNA | (TTCT) | CATTGGACGGTTGGTTCTCT TGGAATTGGAACTTCAAGCA | 274 | TP-GC-YC | 265-285 | 4 | 0.69 |  |

C. annuum
clone similar
to WRKY
transcription
factor 41
듳
C. annuum
4
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0

| E |  |
| :--- | :--- |
| S |  |
|  |  |
|  |  |
| 0 |  |
| 0 | 0 |

C. annuum $\sum_{0}^{4}$ C. annuum
cDNA C. annuum
cDNA
EPMS-715 CK901642
EPMS-716 CO775942

## EPMS-718 CO912216

## EPMS-725 CO910566

EPMS-745 CO907754
EPMS-747 CO907904

|  |  |  |  | SSR development |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-749 | CO909840 | C. annuum cDNA | (AAAACA) <br> 5 | tCCCACGAGTCTtTtTtGAGG AAAGGGAATTATGGGGTTGG | 270 | TP-GC-YC | 268-278 | 5 | 0.65 |  |
| EPMS-750 | CO906574 | C. annuum cDNA | (CCCGAA <br> ) 5 | GGGATCCGAATCAGAATACG TCTAGGTTTCGGGGAGGATT | 253 | TP | 236-257 | 7 | 0.73 | U202027 |
| EPMS-751 | CO776484 | C. annuum cDNA | (CTGCTC) <br> 5 | AGACTGCACCAGCTGAGGTT GTAGCCGCAGACTCCTCAAC | 262 | TP | 213-215 | 2 | 0.44 | U196060 |
| EPMS-755 | CO912244 | C. annuum cDNA | $\left.(\mathrm{A})_{12 \ldots(\mathrm{~T}}\right)_{1}$ | CGCTCGCTACCCTTTCATTA AATTTCGGAAGGGCAAAGAT | 141 | TP-GC-YC | 141-150 | 4 | 0.68 | U204561 |
| EPMS-757 | C0776067 | C. annuum cDNA | $\begin{aligned} & (\mathrm{AG})_{6} \ldots(\mathrm{G} \\ & \mathrm{A})_{9} \end{aligned}$ | CCAACCAAACTTCAACTCCC GATGCACCCACCATTATTCC | 216 | TP-GC-YC | 205-219 | 4 | 0.62 |  |
| EPMS-762 | CO910085 | C. annuum cDNA | $\begin{aligned} & (\mathrm{CT})_{6 \ldots(\mathrm{AT}} \\ & )_{6} \end{aligned}$ | CGGCGAGATATGGACTTGAT CCCACGTTATACCATCCAGG | 261 | TP-GC-YC | 344-357 | 5 | 0.78 |  |
| EPMS-773 | CO909133 | C. annuum cDNA | $(\mathrm{T})_{13 \ldots} \ldots(\mathrm{~T})_{12}$ | CGAGCAACTCCCTCTtATCG TCGAATAGCACGCACGTTAG | 166 | TP | 163-169 | 4 | 0.53 |  |
| EPMS-917 | AY702979 | C. annuum clone YAC genomic sequence | (CT)9 | TTACCCAACCATCAGAAATGG GTGTCCTCTCATGAGCATGG | 176 | TP | 170-178 | 3 | 0.52 |  |


| EPMS-919 | AY702979 |  | (T) ${ }_{12}$ | TTTGGAGTTTGCGAATTGTG AAGCAACGTGTGTAGGGAGG | 231 | TP-GC-YC | 231-238 | 4 | 0.65 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS-921 | AY702979 |  | (A) $1_{13}$ | AAATTTTTGGATTTTGGGCC GCGACTTTAGAAACTTCCGC | 295 | TP | 295-302 | 3 | 0.64 |
| EPMS-922 | AY308828 |  | $(\mathrm{T})_{11} \mathrm{C}(\mathrm{T})_{5}$ | TTTTGGGGTTGTGAAGGAAG TGTGTCTGCACTTTTGAGCC | 492 | TP | 500-515 | 3 | 0.68 |
| EPMS-923 | AY308828 | C. annuum salicylic acidinduced | (A) ${ }_{15}$ | CAAAACCAAATAGGTCCCCC CGCGCAATAATTCAATATCG | 310 | TP-GC | 261-349 | 6 | 0.80 |
| EPMS-924 | AY308828 | protein 19 <br> gene | $\begin{aligned} & (\mathrm{CT})_{6 . .(\mathrm{TA})} \\ & 9 . .(\mathrm{GTA})_{5} \end{aligned}$ | GCCGTCGTCAGAAAAGGTAG tGCATTTCTGTCAGAGGCTG | 287 | TP-GC-YC | 260-295 | 8 | 0.82 |
| EPMS-925 | AY308828 |  | (AT) ${ }_{8}$ | CTCACAAGCAGAAGTGGACC CCCAGTAAAACTTAACCGCAC | 316 | TP | 314-329 | 5 | 0.74 |

${ }^{\text {a }}$ In Italics accessions selected from a cluster of homologous sequences
${ }^{\text {b }}$ SSR useful for mapping studies in the progenies ‘Tabasco' x ‘P4' (TP), 'GF109' x ‘CM334' (GC) or 'Yolo Wonder' x ‘CM 334’ (YC


Fig. 1 UPGMA dendrogram of the 16 genotype set, based on 219 EST-SSR alleles. Sample codes are described in Table 1.

# Chapter 2: A high-resolution intraspecific linkage map of pepper (Capsicum annuum L.) and selection of reduced RIL subsets for fast mapping 

Une carte intra spécifique a haute résolution du piment (Capsicum annuum L.) et sélectionne des sous groupes de RIL pour la cartographie rapide


#### Abstract

A high resolution intraspecific linkage map of pepper (Capsicum annuum L.) was constructed from a population of 297 recombinant inbred lines. The parents were the large-fruited inbred cultivar 'Yolo Wonder' and the hot pepper line 'Criollo de Morelos 334', which is heavily used as a source of resistance to a number of diseases. A set of 587 markers ( 507 AFLPs, 40 SSRs, 19 RFLPs, 17 SSAPs and four STSs) were used to generate the map; of these, 489 were assembled into 49 linkage groups (LGs), including 14 LGs with 10 to 60 markers per LG, and 35 with two to nine markers per LG. The framework map covered 1857 cM with an average inter-marker distance of 5.71 cM . It was possible to assign 23 LGs, composed of $69 \%$ of the markers and covering 1553 cM , to one of the 12 haploid pepper chromosomes, leaving 26 LGs ( 304 cM ) unassigned. The chromosome framework map built with 250 markers led to a high level of mapping confidence and an average inter-marker distance of 6.54 cM . By applying MapPop software, smaller subsets of 141 or 93 most informative individuals were selected with a view to reducing the time and cost of further mapping and phenotyping. To define the smallest number of individuals sufficient for assigning any new marker to a chromosome, subsets from 12 to 45 individuals, and a set of 13 markers distributed over all 12 chromosomes were screened. In most cases, the markers were correctly assigned to their expected chromosome, but the accuracy of the map position decreased as the number of individuals was reduced.


## Introduction

Cultivated Capsicum is native to Central and South America (Pickersgill 1991), and includes the species chinense, baccatum, frutescens, pubescens and annuum. The latter, of Mexican origin, is globally the most widely grown species, and is used as a source of spice and colorant, as a vegetable, and has some medical applications. The species is divided into large-fruited bell peppers and small pungent types (Bosland and Votava 2000). It is diploid, with a somatic chromosome number of 24 , its haploid DNA content is 2.76 pg and its genome size is between 2702 and 3420 Mbp (Arumuganathan and Earle 1993; Galbraith et al. 1983,). Linkage maps have been constructed using both intraspecific annuum populations (Lefebvre et al. 1995; Ben Chaim et al. 2001; Ogundiwin et al.. 2005; Sugita et al. 2005) and interspecific crosses, such as annuum x chinense (Livingstone et al.; 1999; Kang et al. 2001) and annuum x frutescens (Rao et al.2003; Sasvári et al. 2004). Interspecific crosses benefit from a high level of marker polymorphism, but suffer from low fertility, segregation distortion, and major structural rearrangements (Tanksley and Iglesias 1984, Lanteri 1991; Lanteri and Pickersgill 1993), which limit the power of the linkage analysis and restrict their relevance to marker-assisted selection applications (Lefebvre et al. 2002). An integrated map, based on six populations and consisting of 2262 markers covering 1832 cM , has recently been assembled (Paran et al..2004). This has achieved a greatly improved average marker density and better genome coverage. However, confidence in marker position and order remains low, particularly with regard to population-specific markers. Current pepper maps have all been based on a rather low number of individuals per progeny (commonly between 40 and 100, rarely up to 200), resulting in a poor level of resolution (Lefebvre 2005). As the resolution of a genetic map depends on the number of recombination events in the population,
improvement can be achieved by increasing either the number of individuals per progeny and/or the number of markers used to identify recombination events. Mapping in a single population generates a more reliable and accurate map than an integrated map derived from independent mapping populations.
In the present paper, we describe the construction of a high resolution intraspecific genetic linkage map based on a single recombinant inbred line (RIL) population. The map position of highly reproducible, locusspecific, codominant PCR-based markers is of particular value for the integration of genetic information from different populations, and would underpin much applied research in pepper, including gene mapping, QTL analysis, and marker-assisted selection. Finally subsets of the most informative RILs were identified, in order to reduce the genotyping and phenotyping effort. A minimal set of lines was identified to map any new marker to a defined bin (a map segment delimited by recombination events) or to a specific chromosome.

## Materials and methods

## Plant material and DNA extraction

The RIL population was developed at INRA-Montfavet, France, by single seed descent, and comprised 297 F5 progeny from the cross 'Yolo Wonder' x 'Criollo de Morelos 334' (CM334). The parental lines were obtained at INRA-Avigon by 5 to 7 successive selfing from the original local cultivars to generate homozygous inbred lines. The former parent is an American bell pepper inbred line, and the latter a Mexican hot pepper inbred line, used as a source of resistance against Oomycetes
(Phytophthora spp.) (Pochard and Daubèze 1980; Palloix et al. 1988, 1990), nematodes (Meloidogyne spp.) (Djian-Caporalino et al. 1999) and several viruses (Dogimont et al. 1996)

The parents also differ from one another with respect to several key horticultural breeding traits. Seeds of the RIL progenies were obtained by selfing plants under an insect-proof tunnel to avoid outcrossing by insects. Total genomic DNA was extracted from young leaf tissue of each RIL and their parental lines according to CTAB method (Doyle and Doyle, 1990).. DNA quality was checked by $1 \%(w / v)$ agarose gel electrophoresis, and its quantity was assessed by ethidium bromide staining of an agarose gel loaded with DNA samples and DNA standard.

## Molecular markers

## Amplification fragment length polymorphisms (AFLPs)

AFLP reactions were performed as described by Vos et al. (1995), using 19 EcoRI/Msel, 11 Pstl/Msel and 2 Sacl/Msel primer combinations (PCs) (Table 1). The EcoRI, Pstl and Sacl primers were end-labelled with $\left[\gamma-{ }^{33} \mathrm{P}\right]$ dATP to allow fragment visualisation by autoradiography. Segregating markers were encoded by adding to the PC code a threedigit number reflecting the fragment's molecular weight.

## Microsatellites (SSRs)

Forty SSRs were used for mapping purposes (Table 2). Of these, 31 were based on publicly available pepper EST sequences (19 as reported by Nagy et al. (2004, 2006 in preparation), and 12 to be published (Portis et al. unpublished)), with the remaining nine extracted from
genomic libraries Lee et al. (2004). PCR amplifications were performed in a $30 \mu \mathrm{l}$ volume containing 30 ng genomic DNA as template as reported by Nagy et al. (2004). Forward primers were 5'-end labelled with FAM, JOE or NED for analysis on an ABI 3100 sequencer, and with Cy5 for the ALFExpressll platform. Polymorphisms were evaluated by the Genographer (Benham 2001) or by the ALFwin Fragment Analyser (Version 1.03, Amersham Pharmacia Biotech) programs.

## Restriction fragment length polymorphisms (RFLPs)

The 19 RFLP probes used originated from a variety of sources, covering plant cDNAs, gDNAs and known- and unknown function gene sequences (Table 3). These were chosen to allow linkage group (LG) assignment to chromosomes and for ease of map alignment. RFLP procedures have been described elsewhere (Lefebvre et al. 1993).

## Sequence tagged sites (STS)

The four STS markers comprised three converted from purified AFLP fragments and one linked to the Pvr4 resistance gene (Caranta et al. 1999). Amplicons were digested with the appropriate restriction enzyme (Table 3), before separation by $2 \%(\mathrm{w} / \mathrm{v})$ agarose gel electrophoresis.

## Sequence-specific amplication polymorphisms (SSAPs)

The technique first described by Waugh et al. (1997) and adapted by Tam et al. (2005) was employed. SSAP profiling was performed on a subset of 100 of the 297 RILs, using as PCs Tnt1-C00 and T135-E00. For every dominant marker, the letter 'y' (Yolo Wonder) or 'c' (Criollo de Morelos 334 ) indicates the donor parent of the band.

## Data analysis

A $\chi^{2}$ goodness-of-fit test for $1: 1$ segregation was performed for each marker. Co-dominant markers were used to compare the observed and expected (6.25\%) proportion of heterozygosity remaining at F5. Linkage analyses were generated from the RI model of Mapmaker/EXP version 3.0 (Lander et al. 1987). LGs were determined with the "group" command, using as thresholds for linkage detection a minimum LOD of 8 and a maximum recombinant fraction (r) of 0.1 . The sequence of commands was: (i) "order" to determine the most likely marker order within each LG; (ii) "ripple" to confirm marker order using a LOD of 3; (iii) "try" to identify the most likely position of unordered markers within their LG; (iv) "genotype" to identify likely singletons. Putative singletons were checked by re-genotyping and, where necessary, correcting the scoring for markers flanking the apparent recombination event. Recombination fractions were converted into map distances with the Haldane mapping function (Haldane 1919). Ordered LGs were assigned to chromosome by alignment of markers present on prior maps (Lefebvre et al. 2002; Paran et al. 2004). When several LGs where assigned to the same chromosome, "compare" was used to identify the most likely order. The merged groups were validated with "ripple" and a LOD of 3.

## Choice of the most informative individuals

A number (26) of the RILs were removed from the population because of their poor fertility. From the remaining 271 individuals, two subpopulations "A" (141 individuals, i.e., about the half of the original population, and equivalent to one and one-half 96 -well microtitre plates), and "B" (93 individuals, i.e., about one third of the original population,
and equivalent to one 96 -well microtitre plate) were formed with the help of the MapPop software package (Brown and Vision 2000) using the full linkage map as the input file. Seven codominant markers (SSRs EPMS649, EPMS650, EPMS704, EPMS725, EPMS747 and EPMS755 and the STS ASCO02) were used to genotype subpopulation "A" and "B". Smaller subpopulations (ranging from 12 to 45 individuals) were developed in an attempt to define the smallest set of individuals which could chromosomally assign an additional marker. For this purpose, 13 chromosomally defined markers (e32/m55_114y, e44/m61_538c, p14/m39_453y, e44/m61_279y, e38/m61_203y, e31/m58_254c, e38/m60_401y, p17/m39_468c, EPMS310, e38/m60_227y, e31/m58_367y, e38/m60_212y, e43/m54_275c) were employed. The MapPop "sampleexp" command was used to select individuals with a high frequency of recombination events.

## Results

## Marker polymorphism and segregation

A total of 507 AFLPs (Table 1) were used for mapping, of which, respectively, 284, 202 and 21 were generated from EcoRI/Msel, Pstl/Msel, and Sacl/Msel PCs. The EcoRI/Msel PCs generated a mean of 15 scorable polymorphisms, ranging from seven (E33/M56, E40/M49) to 24 (E38/M60). The Pstl/Msel PCs generated a mean of 18 scorable polymorphisms, ranging from 13 (P19/M42) to 29 (P14/M33). The two Sacl/Msel PCs generated 15 (S33/M54) and six (S36/M49) scorable polymorphisms. Among these AFLP markers, 261 originated from 'CM334' and 245 from 'Yolo Wonder'. The SSAP primer sets generated 17 polymorphisms - nine from T135-E00 (five from 'Yolo Wonder', four from 'CM334'), and eight from Tnt1-C00 (five from 'Yolo Wonder', three from 'CM334'). Ninety SSR markers were assayed on DNAs of the
parents and their F1; 40 were polymorphic and thus suitable for mapping (Table 2).

Codominant markers allowed the observed level of heterozygosity to be assessed. A significant excess of heterozygosity was detected at nine loci (maximum 18.7\%), and a deficit at three loci (minimum 0\%). A significant ( $\mathrm{P}<0.01$ ) level of segregation distortion occurred at about $14 \%$ of the loci ( 81 markers), with high ( $\mathrm{P}<0.001$ ) distortion at 45 markers and very high ( $\mathrm{P}<0.0001$ ) distortion at 36 markers. The direction of distortion was towards 'CM334' for 46 loci and towards 'Yolo Wonder' for 35 . About $92 \%$ of the skewed markers were AFLPs, of which 24 were amplified by EcoRI/Msel PCs, 53 by Pstl/Msel PCs and two by Sacl/Msel PCs. The remaining distorted markers were one RFLP and one STS marker. For the linkage analysis, markers showing skewed segregation were classified into: (i) those integrated into the framework map, (ii) those assigned to an LG and placed with the Mapmaker command "try" and (iii) those that were unlinked. As reported in Figure 1, the proportion of skewed markers assigned to the framework map and those placed with the command "try" decreased with the P-value, while in contrast, the proportion of skewed unlinked markers increased as the P-value was reduced. Finally, 24 skewed markers were placed on the framework map, of which 17 were linked to chromosomes (Figure 2).

## Construction of the linkage map and chromosomal assignment

The linkage map was formed from 587 markers ( 507 AFLPs, 40 SSRs, 19 RFLPs, 17 SSAPs and four STSs). Of these, 489 (428 AFLPs, 25 SSRs, 16 RFLPs, 16 SSAPs and four STSs) were distributed on 49 LGs at a LOD of 8 and a maximum $r$ value of 0.1 . Of the LGs, 14 were composed of at least 10 (and up to 60) markers, and 35 carried less than 10 markers. The framework map (Figure 2) used 323 markers
(about 55\% of the total markers) whose order could be confirmed with a LOD of 3 . It covers 1857 cM , with an inter-marker distance of $5.71 \pm 5.70 \mathrm{cM}$. Based on previously published maps (Lefebvre et al. 2002; Paran et al. 2004), 45 markers (32 AFLPs, 12 RFLPs and one STS) allowed for the assignment of 23 LGs to chromosomes. Thus 23 LGs, carrying 69\% of the markers, were allocated to one of the twelve pepper chromosomes (Figure 2). The coverage of this map was 1553 cM, leaving 26 LGs ( 304 cM ) non-assigned. About 77\% (i.e.. 250) of the framework map markers were assigned to pepper chromosomes (223 AFLPs, 10 SSRs, 14 RFLPs, two SSAPs and one STS) which were separated by a mean of $6.54 \pm 6.08 \mathrm{cM}$. Chromosomes P1, P2, P3, P4, P5, P8 and P9 were composed of two or three merged LGs, with the most likely arrangement (LOD>3) shown on Figure 2. Chromosomes P4, P10, and P11 comprised two unmergable LGs. LG23 could not be assigned to chromosome P9, despite carrying the Scar_CD STS locus, which has been recently located on P9 (Djian-Caporalino et al. unpublished) since its location at one or the other extremity of the chromosome was uncertain. The genetic length of the chromosomes with merged LGs (P1, P2, P3, P4, P5, P8 and P9) ranged from 74.8 cM (P6) to 205 cM (P5 and P9).

## Identification of the most informative individuals

The most genetically informative individuals were identified from the two subpopulations " $A$ " ( 141 individuals) and " $B$ "( 93 individuals) to accelerate further mapping. The mean bin size increased from 0.55 cM in the whole RIL population to 0.82 and 1.12 in, respectively, the " $A$ " and " $B$ " subpopulations. Seven further codominant markers were successfully assigned to LGs. Thus, the STS locus ASCO02 was incorporated into the framework map using the "order" command, while six SSRs were successfully placed by the command "try". By using the subpopulation
" $B$ ", these markers mapped to the same LGs but with a lower precision, as a unique marker position could not be identified. The thirteen markers assigned to the 12 chromosomes used to scan the series of smaller subpopulations (12, 24,29 or 45 individuals) were subjected to the "group" command with a LOD score of 3 and an $r$ value of 0.1 . This process allowed for the successful mapping of three (subpopulation size = 12), eight (24), 10 (29) and all 13 (45) markers to their respective expected LGs.

## Discussion

## Molecular markers and map construction

Our aim was to construct a high resolution genetic linkage map of pepper, using a RIL population derived from an intraspecific cross of $C$. annuum. This population is of high relevance to the gene pool used by pepper breeders and should reduce the frequency of skewed segregation, and thereby increase the mapping precision. However, due to the low level of polymorphism (around $40 \%$, see also (Lefebvre et al. 1993, 2001), an extensive genotyping effort was required. The final map has a good level of resolution both because the population is larger than ever used in pepper, and because the use of an F5 generation allowed more meioses to be involved than in the previous F2 or doubled haploid populations. With the 297 RILs, the mapping precision is 0.17 cM ( $r=R /(2-2 R)$ with $R=1 / 297$ ) compared to 0.34 cM (Lefebvre et al. 1995) for a similarly sized DH population. Because of the high percentage of homozygosity, an immortalised RIL population allows for the replication of phenotypic assays across different environments to supply robust estimates of trait means, thus making the identification of quantitative trait loci more efficient and reliable.

About $14 \%$ of the markers showed distorted segregation ratios - higher than the $7 \%$ to $10 \%$ previously reported in the intraspecific maps of Lefebvre et al. (2002) and Sugita et al. (2005), but considerably lower than levels reported in interspecific maps (Livingstone et al. 1999; Kang et al. 2001). Although about $90 \%$ of the distortion observed in doubled haploid populations generated with 'CM334' as a parent involved a deficit of 'CM334' alleles) Lefebvre et al. 1995), however, more markers were skewed towards 'CM334' than towards 'Yolo Wonder' in the RIL progeny. A possible cause for this inconsistency is that 'CM334' is strongly recalcitrant to in vitro androgenesis (unpublished data), and thus its use here as a parent of a single seed descent-derived population avoids any in vitro selection against 'CM334' alleles. The higher than expected level of heterozygosity may reflect selection against homozygosity for some 'CM334’ alleles at some loci.

In order to increase confidence in marker placement, a stringent threshold was applied (LOD $>8$ and $\mathrm{r}<0.1$ ). This resulted in the definition of 49 LGs, of which 21 included only a limited number of loci, and presumably will be merged into larger groups with the addition of further markers. The application of less restrictive conditions allows the generation of fewer LGs, but significantly decreases the level of confidence. One large LG carrying markers known to be located on distinct chromosomes was found at $\mathrm{r}<0.2$. Unassigned LGs, together with some unlinked markers, could be associated with various chromosomes at a LOD $<8$ and $r>0.1$, but their position and order was not precisely determinable, and thus they were not placed on the framework map. About $55 \%$ of the whole marker set was usable as framework markers, as their map order was confirmed at LOD > 3.0. The framework markers were evenly spaced, although some clustering was evident on chromosomes P5, P9 and P11. Clustering is a common phenomemon, particularly, although not exclusively, in centromeric regions. It is thought to arise as a result of an inhibition of recombination
in these regions (Boersma et al. 2005) The overall length of the map is 1857 cM , similar to the 1832 cM reported for the integrated map of Paran et al. (2004), but significantly greater than the 1466 cM reported recently by Ogundiwin et al. (2005) for a pepper map based on RILs. The good genome coverage of the present map, calculated to be $86.5 \%$, according to the method of Lange and Boehnke (1982), derives from the use of a large number of markers.

## Map comparison

We have compared the framework map with those of Lefebvre et al. (2002) and Paran et al. (2004). Based on 32 shared markers, 23 LGs could be aligned with regions on the pre-existing maps. All common AFLP markers mapped to the same location, establishing their locusspecificity (Rouppe van der Voort et al. 1997; Lefebvre et al. 2002). The alignment was successful for all the chromosomes except P1 and P8. Chromosome P1 of the integrated map was split into two LGs in both the present map, and that of Lefebvre et al. (2002). P1 is colinear with the upper part, and P8 colinear with the lower part of the integrated LG1. Chromosome P8 of the integrated map aligns with the intraspecific chromosome P1 of Lefebvre et al. (2002), although it has not been possible to make an alignment with any of the present LGs due to a lack of shared markers. These results confirm the suggestion, made by Pickersgill (1991), of a reciprocal chromosomal translocation between annuum and chinense, which could be responsible for pseudolinkage between loci close to the interchange breakpoint. This breakpoint might be localised at 75 cM of LG1 of the integrated map of Paran et al. (2004), which corresponds to the lower part of the intraspecific chromosome P8.

## IDENTIFICATION OF THE MOST INFORMATIVE RILS AND SELECTIVE GENOTYPING

As reported by Isidore et al. (2003), the larger the population, the greater the number of meioses upon which the map can be based, and so the greater the number of markers which can be ordered. However, the genotyping and phenotyping of large populations is costly. As a way of reducing this cost, we have sought to identify the most genetically informative individuals for further phenotypic and mapping studies. The MapPop software (Brown and Vision 2000) is designed, on the basis of an already developed map, to identify optimized subpopulations which include the most informative individuals. Among the RILs, four subpopulations were identified, consisting of 141, 93, 45 or 29 individuals. When their potential for mapping was tested, both larger subpopulations delivered reasonable mapping precision, with a higher precision achieved when the largest subpopulation was genotyped. In the 45 individual subpopulation, the mean bin size increased to 2.00 , and all of the 13 markers used for the test were precisely assigned to a chromosome. In the 29 individual subpopulation, the mean bin size increased to 2.83 , and 10 out of the 13 markers were assigned to the expected chromosome, with an imprecise location within the chromosome. This rather small subset was identified to allow for a rapid screen of new markers and to assign them to a LG with the minimum of laboratory work, as proposed by Xu et al. (2005). By applying this method, Howad et al. (2005) identified a subset of six lines out of the 88 used to construct a Prunus reference map. For further mapping saturation, only RIL individuals belonging the subpopulation "A" will be selected, a strategy which should reduce to half the cost and time required for genotyping. Our current results, as previously demonstrated by Xu et al. (2005), show that selective genotyping is an attractive strategy in situations requiring phenotypic testing and QTL mapping.

Note: The complete map involving all 489 markers assigned to LGs is available at http://www.inra.avignon.fr, and the subset of the 45 most informative RILs is available on request from alain.palloix@avignon.inra.fr.
Pepper intra-specific genetic linkage map - Carte du piment intra spécifique
Table 1. Number of polymorphic bands generated by each AFLP primer combination.

| Primers <br> combination | Number of <br> polymorphic bands | Primers <br> combination | Number of <br> polymorphic bands |
| :---: | :---: | :---: | :---: |
| S33/M54 | 15 | E42/M48 | 19 |
| S36/M49 | 6 | E43/M53 | 13 |
| E31/M58 | 18 | E43/M54 | 13 |
| E32/M55 | 10 | E44/M51 | 14 |
| E33/M56 | 7 | E44/M61 | 12 |
| E34/M53 | 16 | P11/M49 | 14 |
| E36/M47 | 14 | P14/M33 | 29 |
| E36/M52 | 19 | P14/M39 | 27 |
| E37/M54 | 9 | P14/M41 | 21 |
| E38/M60 | 24 | P15/M40 | 16 |
| E38/M61 | 18 | P15/M43 | 17 |
| E40/M47 | 18 | P17/M32 | 16 |
| E40/M49 | 7 | P17/M39 | 11 |
| E41/M48 | 20 | P19/M42 | 14 |
| E41/M54 | 19 | P19/M45 | 18 |
| E41/M61 | 16 | P25/M42 | 19 |

Pepper intra-specific genetic linkage map - Carte du piment intra spécifique
Table 2. SSR primers and the molecular weight of amplicons. * SSRs from Nagy et al. (2004); ** SSRs from
Portiset al. (unpublished).

| SSR | GenBank accession nro | Size (bp) | SSR | GenBank accession $\mathrm{nr}^{\circ}$ | Size (bp) | SSR | GenBank accession nro | Size (bp) |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| EPMS310* | BM061162 | 300/330 | EPMS429* | CA525246 | 174/162 | EPMS747** | CO907904 | 280 |
| EPMS340* | BM064640 | 273/260 | EPMS441* | CA847465 | 118/124 | EPMS755** | C0912244 | 150/140 |
| EPMS353* | BM067271 | 299/305 | EPMS472* | BM061910 | 293/320 | EPMS919** | AY702979 | 231 |
| EPMS372* | CA515633 | 326/324 | EPMS480* | BM062655 | 256/253 | Hpms 1_1 | Lee et al. 2004 | 283 |
| EPMS376* | CA516334 | 260/256 | EPMS497* | CA522759 | 251/250 | Hpms 1_111 | Lee et al. 2004 | 159 |
| EPMS377* | CA516439 | 156/162 | EPMS561** | BM068460 | 114/117 | Hpms 1_172 | Lee et al. 2004 | 344 |
| EPMS386* | CA517699 | 149/143 | EPMS643** | CO908387 | 212 | Hpms 1_214 | Lee et al. 2004 | 100 |
| EPMS391* | CA519548 | 187/185 | EPMS649** | C0776131_c43 | 260/240 | Hpms 1_5 | Lee et al. 2004 | 311 |
| EPMS402* | CA523427 | 203/201 | EPMS650** | CO910134 | 250 | Hpms 1_62 | Lee et al. 2004 | 186 |
| EPMS409* | CA525246 | 174/164 | EPMS670** | C0910307 | 102 | Hpms 2-13 | Lee et al. 2004 | 189 |
| EPMS410* | CA525390 | 187/184 | EPMS686** | CK901658 | 158 | Hpms 2_24 | Lee et al. 2004 | 205 |
| EPMS419* | CA523208 | 226/224 | EPMS694** | CO912323 | 238 | Hpms1-69 | Lee et al. 2004 | 217 |
| EPMS421* | CA514272 | 256/247 | EPMS704** | C0910889 | 160/150 |  |  |  |
| EPMS426* | CA517376 | 116/108 | EPMS725** | C0910566 | 158 |  |  |  |

Table 3. Chromosome assignment and function (where known) of RFLP and STS markers.

| RFLP probe | Description and Putative function (species) | Species of origin of the sequence for RFLP | Reference of the sequence | Pepper Chromosome |
| :---: | :---: | :---: | :---: | :---: |
| CD035 | Hypothetical yeast protein / yeast | Tomato | Ganal et al. 1988 | P2 |
| CT080 | Serine Hydroxymethyl Transferase / Arabidopsis thaliana | Tomato | Ganal et al. 1988 | P5 |
| CT100 | Xyloglucan endo-1,4-beta-D-glucanase / Tomato | Tomato | Ganal et al. 1988 | P9 |
| CT145 | G subunit of the Vacuolar-type AATPase / Tobacco | Tomato | Lefebvre et al. 2002 | P9 |
| CT224 | Alpha amylase / Potato | Tomato | Ganal et al. 1988 | P4 |
| GC015_1 | Acid class III chitinase-lysozyme / Tobacco | Tobacco | Stintzi et al. 1993 | P5 |
| GC207 | Dehydration-induced protein (Clone LEEB'7-2) / Arabidopsis thaliana | Tomato | Causse et al. 2004 | P4 |
| PG101 | pepper genomic DNA | Pepper | Livingstone et al. 1999 | P3 |
| PG108 | pepper genomic DNA | Pepper | Livingstone et al. 1999 | P6 |
| PT001c | AFLP fragment C45/M51_430.4 from the 'RH' genotype / Potato | Potato | unpublished data from the INRA laboratory | P2 |
| T_1430 | Putative phospholipase / Arabidopsis thaliana | Tomato | http://www.sgn.cornell.edu/ | P5 |
| TG029 | Subtilisin-like proteinase (EC 3.4.21.-) 4 / Tomato | Tomato | http://www.sgn.cornell.edu/ | P1 |
| TG046 | unknown protein / Arabidopsis thaliana | Tomato | http://www.sgn.cornell.edu/ | unlinked |
| TG123 | Phosphatidylglycerolphosphate synthase (PGP1)/ Arabidopsis thaliana | Tomato | http://www.sgn.cornell.edu/ | P5 |
| TG132 | S-locus protein kinase family / Arabidopsis thaliana | Tomato | http://www.sgn.cornell.edu/ | P4 |
| TG281 | Guanine nucleotide-exchange protein-like / Arabidopsis thaliana | Tomato | http://www.sgn.cornell.edu/ | P8 |


| Pepper intra-specific genetic linkage map - Carte du piment intra spécifique |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
| $\begin{gathered} \text { C2_At3g52220 } \\ \text { C2_At3g58470 } \\ \text { MPG004 } \end{gathered}$ | unknown protein / Arabidopsis thaliana unknown protein / Arabidopsis thaliana AFLP fragment E40/M47_210c from the 'CM334' genotype / Pepper | Tomato <br> Tomato <br> Pepper | http://www.sgn.cornell.edu/ <br> http://www.sgn.cornell.edu/ unpublished data from the INRA laboratory | P12 <br> unlinked <br> P12 |
| STS | Description | Species of origin of the sequence | Reference |  |
| ASC002 | CAPS marker linked to Pvr4 in pepper / A/wNI | Pepper | Caranta et al. 1999 | P10 |
| Scar_CD | SCAR marker linked to Me cluster in pepper | Pepper | Djian-Caporalino et al. Unpublished ASC60F 5'-GAAGCTTATGTGGTAMCC3' ASC60R 5'-GCAAAGTAATTATATGCAAGAGT-3' | LG23 |
| ASC075 | CAPS marker / Ddel | Pepper | ASC75F 5'- <br> TAACAAACATATGGTGAGCATGAA-3' ASC75R 5'- <br> GTAGGCGGCTAGAGGTTAGGT-3' | P7 |
| Z478 | CAPS marker / HindllI | Pepper | Z478F 5'- <br> GGAAAGTGCACTCACGAACA-3' <br> Z478R 5'- <br> CCCCATTCATCCATACGAAA-3' | LG48 |

[^0]
Figure1. Proportion of skewed markers in the three classes ( $i$ - markers included in the framework map, ii-
markers assigned to LGs and placed with the Mapmaker command "try" and iii- unlinked markers) and
distribution $\quad$ according $\quad$ to $\quad$ P-value.
Pepper intra-specific genetic linkage map - Carte du piment intra spécifique


Pepper intra-specific genetic linkage map - Carte du piment intra spécifique

Pepper intra-specific genetic linkage map - Carte du piment intra spécifique

| LG39 | LG40 | LG41 | LG42 | LG44 |
| :---: | :---: | :---: | :---: | :---: |
|  |  | $\underset{2,8}{0,0} \mathrm{O}_{\mathrm{p}}^{\mathrm{p} 19 / m 42 \_874 \mathrm{y}}$ | $\underset{7,0-10-p 25 / \mathrm{m} 45 \_335 \mathrm{c}}{\mathrm{p} 14 / \mathrm{m} 39 \_345 \mathrm{c}}$ | $\begin{gathered} 0,0-\text { - p1 1/m39_195c } \\ 6,511 / \mathrm{m} 49 \_105 y \end{gathered}$ |
| LG45 | LG46 | LG47 | LG48 | LG50 |
|  |  | $\underset{1,9}{0,0} \bigodot_{\mathrm{p} 14 / \mathrm{m} 33_{-} 313 \mathrm{y}}^{\mathrm{p} 14 / \mathrm{m} 31 \mathrm{c}}$ | ${ }_{10,0}^{0,0} \int_{\text {Z478 }}^{\text {c33/m54_201y }}$ |  |
| LG51 |  |  |  |  |
|  |  |  |  |  |

[^1]
# Chapter 3: QTLs analysis of 13 horticultural traits in three intra-specific pepper populations. Allele conservation and diversity in pepper, macro-synteny in the Solanaceae family. 

Analyse QTL de 13 caractères horticulturaux dans trios populations de piment intra spécifiques. Conservation des allèles et diversité dans le piment, macro synténie dans la famille des Solanaceae.


#### Abstract

QTL analysis of 13 horticultural traits (i.e fruit and plant traits) was performed on 3 intra-specific populations of pepper (C. annuum) originating from the crosses: Yolo Wonder $X$ Criollo de Morelos (YC), Perennial X Yolo Wonder (PY) and H3 X Vania (HV). A TOTAL OF 78, 24 AND 30 QTLS WERE DETECTED FOR THE YC, PY AND HV POPULATIONS RESPECTIVELY WITH A P LEVEL OF 0.05 AS THE THRESHOLD TO DECLARE THE PRESENCE OF A QTL. QTL comparison among the three populations revealed that 9 QTLs were located in the same genomic regions, while 11 were detected in only two of the three populations considered, i.e. 3 in YC and PY populations, 4 in PY and HV and 4 in YC and HV. AN ANALYSIS OF SYNTENY AMONG TOMATO, PEPPER, POTATO AND EGGPLANT FOR COMMON GENOMIC REGIONS CONTROLLING HORTICULTURAL TRAITS WAS CARRIED OUT. PUTATIVE ORTHOLOGOUS REGIONS WERE FOUND, IN PARTICULAR FOR FRUIT WEIGHT, FRUIT LENGTH, FRUIT DIAMETER, FRUIT SHAPE, FLOWERING EARLINESS AND PLANT HEIGHT, WHICH MAY HAVE BEEN CONSERVED DURING EVOLUTION AND INDEPENDENT SELECTION PERFORMED ON THESE SPECIES

Through selective genotyping, by means of MapPop application, subsets of 141 and 93 individuals of the YC population were identified, as being the most informative for future mapping studies and phenotypic tests. The subsets were tested for QTL mapping accuracy of two horticultural and one resistance traits. As expected the number of QTLs detected decreased with the


QTLs analysis of horticultural traits - Analyse QT des caractères horticulturaux
reduction of population size, anyhow, selective genotyping leads to more informative results than the use of random samples.

## Introduction

Pepper, like most cultivated crops, is subjected to intense breeding efforts by Institutes and private companies around the world. Breeders have to combine favourable alleles controlling climatic adaptation, plant architecture, fruit characteristics and disease resistance in each cultivar. This multitrait selection may benefit from favourable linkage between alleles, but unfortunately is often slow down by unfavourable linkages. In pepper, disease resistances are generally introduced from small fruited and pungent local populations into genetically distant elite genitors; moreover, cultivar types strongly differ in plant ideotypes and fruit shapes. This double constraint "multiple ideotypes and exotic germplasm resources" motivates an integrated analysis of traits conferring horticultural characteristics and disease resistance in order to identify possible barriers (unfavourable linkages) to genotype construction.
Previous studies report on QTL identification of fruit-related traits in intraspecific (Ben Chaim et al. 2001) and inter-specific crosses (Rao et al. 2003, Zygier et al. 2005); while a few information are available on QTLs controlling plant height (Ben Chaim et al. 2001a) and flowering time (Rao et al. 2003)
Comparative genome analysis was carried out in Solanaceae; synteny relationships were explored by using tomato-derived probes in the four most important species: pepper, tomato, potato and eggplant (Tanksley et al. 1992, Livingstone et al. 1999, Doganlar et al. 2002). Accurate studies on conserved regions affecting pest resistance genes were performed by Grube et al (2000b). Syntenic studies on horticultural traits were done by comparing pepper and tomato (Ben Chaim et al. 2001a, Rao et al. 2003) and, more recently, by comparing pepper, tomato and eggplant (Doganlar et al. 2002, Frary et al. 2003) allowing to better
elucidate the genomic organization of Solanaceae. Results showed that some domestication-related traits, like fruit weight and the fruit shape, have been conserved during the evolution and the domestication within the Solanaceae family. Notwithstanding, orthologous QTLs show differences in effect between species.

In this chapter the results on QTL analysis performed on 13 horticultural traits which control both fruit and plant characteristics are reported. The recombinant inbred population previously used to develop the genetic linkage map (chapter 2) was characterized for the horticultural traits. The QTL analysis was firstly performed in this whole progeny. Two other pepper populations originated from the crosses Perennial $X$ Yolo Wonder and H3 X Vania were analysed with the objective to explore the conservation or diversity of QTLs for horticultural traits in an enlarged pepper germplasm. Synteny analyses among related species, i.e. pepper, tomato, potato and eggplant were also carried out for identifying conserved genomic regions which influence horticultural traits.
Lastly, in this chapter are reported the results of QTLs analysis carried out by using the two previously identified subpopulations (chapter 2) sampled from the large recombinant inbred progeny thanks to the application of the MapPop software (Brown and Vision, 2000).

## Materials and Methods

## Plant material and trait evaluation

The plant material consisted of three intra-specific populations developed from crosses between large fruited and sweet pepper inbred lines ('Yolo Wonder' or 'Vania') and small fruited pungent inbred lines derived from local cultivars from Mexico (CM334), India (Perennial) and East Africa (H3) (Figure 1). These populations were developed at INRAMontfavet, France, and were previously used for genetic analysis of disease resistance traits (Daubèze et al. 1995, Lefebvre et al. 1995, Bonnet et al. submitted). The YC population included 297 recombinant inbred lines (RIL:F6) which were developed by selfing from the F5 RIL population used for map development and obtained by the F1 hybrid "Yolo Wonder" x "Criollo de Morelos 334" (CM334).
The PY population counted114 doubled haploids progeny derived from in vitro androgenesis of the F1 hybrid "Perennial" x "Yolo Wonder". The HV population consisted of 101 doubled haploids progeny obtained from the F1 hybrid "H3" x "Vania".
For trait evaluation, the four parents, the F1 hybrids and their respective progenies were analysed. The experiments were arranged in a randomized complete block design with three replications. Each replication was constituted from 3 to 6 plants from each inbred or doubled haploid line, depending on the progeny. The parents and the F1 hybrids were also included as control genotypes in all the experiments.
A total of 13 horticultural key breeding traits were measured, which are also used as morphological descriptors of pepper accessions in international germplasm collection: (1) the mean fruit weight transformed into its logarithm value (Lfw), (2) the fruit length (Frl) - the distance (mm) from the pedicel attachment to the apex, (3) the fruit diameter (Frd) - the
maximum fruit width (mm), (4) the fruit shape (Frs) - calculated by the ratio fruit length / fruit diameter, (5) the pericarp thickness (Pet) - (mm), (6) the number of fruit locules (No), (7) the pedicel length (Pel) (mm), (8) the flowering earliness (Flw) - numbers of days from the sowing date to the anthesis of the first flower, (9) Axis length (Axl) - the length of the primary axis (cm) from the cotyledons to the first branching, (10) the number of leaves developed on the primary axis (between the cotyledons and first branching) (Nle), (11) the mean internode length (InI) - the ratio of the total axis length to the number of leaves (cm), (12) the mean internode growth time (day) (Int) - the ratio of the flowering time minus 15 days (time necessary for cotyledon development) to the number of leaves and (13) the axis growth speed (Axs) (mm/day) which is the ratio of the axis length to the flowering time minus 15 days. For the RILs-F6 progenies, data from all the 13 traits were available, while for the PY and the HV progenies, data for ten and eleven traits were available respectively (Table 1).

## Yolo Wonder



CM 334
Perennial

## Vania H3



Figure 1: fruits of the pepper parental lines.

| Trait analysed |  | Population |  |  |
| :---: | :---: | :---: | :---: | :---: |
|  |  | YC | PY | HV |
| Logarithm of fruit weight | Lfw | $X$ | $X$ | X |
| Fruit length | Frl | $X$ | $X$ | X |
| Fruit diameter | Frd | $X$ | $X$ | X |
| Fruit shape | Frs | $X$ | $X$ | X |
| Pericarp thickness | Pet | $X$ | na | na |
| Number of locules | Nlo | $X$ | na | na |
| Peduncule length | Pel | $X$ | na | X |
| Flowering earliness | Flw | $X$ | $X$ | X |
| Axis lenght | Axl | $X$ | $X$ | X |
| Number of leaves | Nle | X | $X$ | X |
| Internode length | Inl | $X$ | $X$ | X |
| Internode growth time | Int | $X$ | $X$ | X |
| Axis growth speed | Axs | $X$ | $X$ | X |

Table1 Traits analysed for QTL analyses. The cross $X$ is used for indicating where trait data were available in each population and na for data not available.

## Data analyses

All statistical analyses were performed with the $R$ software package ( $R$ Development Core Team 2006). Descriptive statistic analyses were performed to evaluate the goodness of the phenotypic data from the three populations: distribution analyses, median/mean/variance.

Analysis of variance (ANOVA) was applied to estimate genotypic/environmental effects according to the model
$y_{i j}=\mu+b_{j}+g_{i}+\varepsilon_{i j}$,
where $\mu, \mathrm{b}_{\mathrm{j}}, \mathrm{g}_{\mathrm{i}}$ and $\mathrm{e}_{\mathrm{ij}}$ are the grand mean, block effect, genotype effect and error effect, respectively. Narrow-sense heritability $\left(h^{2}\right)$ values were calculated using the formula $h_{n}^{2}=\sigma_{A}^{2} /\left(\sigma_{A}^{2}+\sigma_{E}^{2} / n\right)$ where $\sigma_{A}^{2}$ is the genetic variance and $\sigma_{E}^{2}$ is the environmental variance (including block, interaction and error effects) and n the number of repeats (blocks). Pearson correlations between traits were estimated.

## Map construction and QTL analyses

Genetic linkage maps used for QTLs analysis were the one previously reported in chapter 2 (Barchi et al. in press) for the F5YC progeny and the ones reported by Lefebvre et al. (2002) for the PY and HV populations.
QTL analysis were performed with QTL Cartographer software (Basten et al. 2002) using both interval mapping (IM) (Lander and Botstein, 1989) and composite interval mapping (CIM) (Zeng,1994). A total of 1,000 permutations for both methods to establish empirical LOD thresholds at the $5 \%$ probability level (Churchill and Doerge, 1994) were performed. The proportion of observed phenotypic variation attributable
to a particular QTL was estimated by the coefficient of determination $\left(R^{2}\right)$. The total phenotypic variation explained was estimated by fitting a linear model (multiple regression) including all putative QTL for the respective trait simultaneously. For each QTL, the marker with the highest LOD was included as explicative variable in the multiple regression. Digenic interactions between all the markers of the core maps were tested with each trait using a two-way ANOVA, as described by Lefebvre and Palloix (1996). Significant epistasis was retained when $\mathrm{P}<10^{-6}$.

QTLs were named as follow: the three first letters indicate the trait analysed. The first following number indicate the chromosome or the linkage group (LG) on which the QTL was detected and the second number indicate the position in which the QTL was identified in the studied progeny. QTLs bearing the same name in distinct progenies does not mean identity and will be further discussed. Finally the code IM (Interval Mapping) or CIM (Composite Interval Mapping) was added when QTLs were detected with a single of these 2 methods.

## Choice of the most informative individuals in the YC population

A number (26) of the RILs were removed from the population because of their poor fertility. From the remaining 271 individuals, two subpopulations "A" (141 individuals, i.e., about the half of the original population), and "B" (93 individuals, i.e., about one third of the original population) were selected with the help of the MapPop software package (Brown and Vision 2000) using the full linkage map as the input file (see chapter 2). The two subsets were tested for QTL mapping accuracy for three traits: (i) Lfw (logarithm of fruit weight), (ii) AxI (plant axis length) and (iii) Rec (receptivity) which is a component of resistance to Phytophthora capsici (data from Bonnet et al. Submitted).

## Results

## Phenotypic variation and trait correlations

## ii. Yolo Wonder X Criollo de Morelos population

Mean phenotypic values and narrow-sense heritability estimates for each trait in study are listed in Table 2. The large fruited Yolo Wonder produces blocky fruits which are heavier, longer, larger and with a thicker pericarp than the ones from Criollo the Morelos. The YW fruit shape was close to 1 while CM334 produced elongated fruits, its number of locules was always 4 while varied from to 2 to 3 for CM334. The pedicel length of both parents was similar. Considering plant traits, Yolo Wonder displayed a later flowering date, a shorter primary axis than CM334 but an analogous number of leaves on the axis; this resulted in a slower axis growth speed, a slightly shorter internode length and slightly slower internode growth time. The F6 progeny displayed normal-like distributions for all the traits (Figure 2). Most of the F6 inbred lines displayed intermediate values between the parental lines for all the fruit traits, except pedicel length for which transgressive phenotypes (ie. values outside of the parental range) were observed as the average value was higher than that both parental lines. Considering plant traits, the F6 inbred lines frequently displayed transgressive values, particularly when the parental values were close to each-other (number of leaves, internode length, internode growth time).
Heritability was high for each trait, ranging from 0.84 for the number of locules to 0.97 for fruit diameter and fruit shape (Table 2).

Strong positive or negative correlations were found between fruit traits as well as between plant traits (table 3), but no significant ( $\mathrm{p}<0.05$ ) or low correlation coefficient (<0.3) were detected between these two types of traits. The fruit weight increased with the fruit length, fruit diameter
and pericarp thickness, and the fruit diameter with the pericarp thickness and the number of locules. A positive correlation was also detected between fruit length and pedicel length. Negative correlations were detected between the fruit shape and the pericarp thickness and the number of locules. Other correlations resulted from dependant variables (the fruit shape resulting from the ratio Frl/Frd). Considering plant traits, a late flowering date was associated with a high number of leaves developed on the axis and a long primary axis. One may also notice that a long axis and a high number of leaves was associated with a higher speed of axis growth (and a higher speed of internode growth).




Figure 2: Frequency distribution for the 13 traits analysed in the YC population: logarithm of the mean fruit weight value (Lfw), the fruit length (Frl), the fruit diameter (Frd), the fruit shape (Frs), the pericarp thickness (Pet), the number of fruit locules (Nlo), the pedicel length (Pel), the flowering earliness(flw), Axis length (AxI), the number of leaves ( Nle ), the mean internode length (InI), the mean internode growth time (Int), the axis growth speed (Axs). Parental mean values are designated CM334 (Criolo de Morelos), YW (Yolo Wonder) while F1 indicates the hybrid mean.
QTLs analysis on horticultural traits

| Trait |  | Mean | Mean | Mean | Mean | Heritability |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | Yolo Wonder | Criollo de Morelos | F1 | F6 |  |
| Logarith of fruit weight | Lfw | 5.38 | 1.83 | 3.27 | 3.33 | 0.96 |
| Fruit length (mm) | Frl | 91.47 | 58.83 | 81.88 | 77.83 | 0.96 |
| Fruit diameter (mm) | Frd | 77.83 | 14.87 | 29.98 | 36.98 | 0.97 |
| Fruit shape | Frs | 1.02 | 3.17 | 2.2 | 2.19 | 0.97 |
| Pericarp thickness (mm) | Pet | 7.45 | 3.68 | 2.49 | 4.06 | 0.92 |
| Number of locules | Nle | 4 | 2.29 | 2.46 | 2.85 | 0.84 |
| Pedicel length (mm) | Pel | 41 | 38.23 | 49.94 | 47.63 | 0.95 |
| Flowering (days) | Flw | 60.96 | 53.07 | 52.73 | 56.62 | 0.88 |
| Axis length (cm) | AxI | 18.01 | 22.82 | 25.1 | 22.06 | 0.94 |
| Number of leaves | Nle | 12.12 | 12.44 | 11.26 | 11.56 | 0.92 |
| Internode length (cm) | InI | 1.49 | 1.85 | 2.25 | 1.93 | 0.91 |
| Internode growth time (days) | Int | 3.86 | 3.12 | 3.39 | 3.68 | 0.9 |
| Axis growth speed (mm/day) | Axs | 3.93 | 6.01 | 6.66 | 5.32 | 0.93 |

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QTLs analysis on horticultural traits

| Trait | Lfw | Frl | Frd | Frs | Pet | No | Pel | Flw | AxI | Nle | Inl | Int | Axs |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Lfw |  | 0.41 | 0.87 | -0.31 | 0.73 | 0.23 | 0.14 | 0.23 | -0.09 | -0.16 | 0.05 | 0.28 | -0.17 |
| Frl |  |  | 0.06 | 0.69 | 0.06 | -0.29 | 0.40 | 0.03 | 0.03 | -0.11 | 0.14 | 0.12 | 0.03 |
| Frd |  |  |  | -0.64 | 0.68 | 0.36 | -0.02 | 0.26 | -0.11 | -0.13 | 0.00 | 0.27 | -0.20 |
| Frs |  |  |  |  | -0.43 | -0.46 | 0.30 | -0.14 | 0.08 | 0.01 | 0.09 | -0.09 | 0.14 |
| Pet |  |  |  |  |  | 0.17 | -0.04 | 0.20 | -0.03 | -0.10 | 0.06 | 0.20 | -0.10 |
| Nlo |  |  |  |  |  |  | -0.22 | 0.03 | 0.02 | 0.06 | -0.05 | -0.05 | 0.01 |
| Pel |  |  |  |  |  |  |  | 0.25 | 0.08 | -0.13 | 0.21 | 0.26 | -0.01 |
| Flw |  |  |  |  |  |  |  |  | 0.26 | 0.34 | 0.01 | 0.12 | -0.08 |
| AxI |  |  |  |  |  |  |  |  |  | 0.50 | 0.66 | -0.39 | 0.94 |
| Nle |  |  |  |  |  |  |  |  |  |  | -0.30 | -0.88 | 0.40 |
| Inl |  |  |  |  |  |  |  |  |  |  |  | 0.34 | 0.68 |
| Int |  |  |  |  |  |  |  |  |  |  |  |  | -0.45 |
| Axs |  |  |  |  |  |  |  |  |  |  |  |  |  |

positive and $>0.3$
Table 3: Pearson correlation coefficient between traits measured in the F6 progeny. Traits analysed are logarithm of the mean fruit weight value (Lfw), the fruit length (Frl), the fruit diameter (Frd), the fruit shape (Frs), the pericarp thickness (Pet), the number of fruit locules (Nlo), the pedicel length (Pel), the flowering earliness(flw), Axis length (Axl), the number of leaves (Nle), the mean internode length (lnl), the mean internode growth time (Int), the axis growth speed (Axs). Coloured boxes indicate significant correlations $(P>0.05)$

## iii. Perennial X Yolo Wonder population

Mean phenotypic value estimates for each trait are listed in Table 3. Correlations between traits are presented in Table 4. Differences between the parental lines are close to those observed in the previously described cross, taking into account that Perennial fruits are even lighter and narrower than CM334. Differences between parents for plant traits are stronger since Perennial displayed a longer primary stem, a higher number of leaves, and a longer flowering time than Yolo Wonder. The PY progeny displayed normal-like distributions for all the traits. The PY doubled haploid lines displayed intermediate values between the parental lines for all the fruit traits. The PY lines frequently displayed transgressive values, particularly when the parental values were close to each-other (Flw, Axl, Int and Axs) (Figure 3).
Narrow sense heritability were high for all the traits, ranging from 0.87 for flowering time to 0.97 for fruit shape (Table 4).
As in the YC population a significant positive correlation was detected between fruit weight, length and diameter, as well as between the flowering time, axis length, number of leaves. Negative correlations were found for Axl and Int, Nle with Int and for Int and Axs.
Contrarily to the YC progeny, significant negative correlations ( $p<0.05$ ) were found between fruit and plant traits, particularly, the fruit weight, length and diameter were found to decrease when the lateness of flowering, the axis length and the number of leaves increased. (Table 3).


QTLs analysis on horticultural traits



Figure 3: Frequency distribution for the 10 traits analysed in the PY population: logarithm of the mean fruit weight value (Lfw), the fruit length (Frl), the fruit diameter (Frd), the fruit shape (Frs), the flowering earliness(flw), Axis length (Axl), the number of leaves ( Nle ), the mean internode length ( InI ), the mean internode growth time (Int) , the axis growth speed (Axs). Parental mean values are designated Perennial (Perennial), YW (Yolo Wonder) while F1 indicates the hybrid mean.

QTLs analysis on horticultural traits

|  |  | FrI | Frd | Frs | Flw | Ax | Nle | InI | Int | Axs |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Lfw |  | 0,52 | 0,90 | -0,31 | -0,47 | -0,50 | -0,47 | -0,38 | 0,40 | -0,48 |
| Frl |  |  | 0,27 | 0,59 | -0,52 | -0,49 | -0,53 | -0,24 | 0,46 | -0,42 |
| Frd |  |  |  | -0,57 | -0,42 | -0,43 | -0,38 | -0,38 | 0,32 | -0,42 |
| Frs |  |  |  |  | -0,11 | -0,09 | -0,17 | 0,09 | 0,16 | -0,05 |
| Flw |  |  |  |  |  | 0,73 | 0,79 | 0,38 | -0,52 | 0,58 |
| AxI |  |  |  |  |  |  | 0,82 | 0,80 | -0,71 | 0,98 |
| Nle |  |  |  |  |  |  |  | 0,34 | -0,90 | 0,75 |
| Inl |  |  |  |  |  |  |  |  | -0,27 | 0,85 |
| Int |  |  |  |  |  |  |  |  |  | -0,70 |
| Axs |  |  |  |  |  |  |  |  |  |  |
|  | positive negativ | and and |  |  |  |  |  |  |  |  |

Table 5: Correlation between traits in the PY population: Traits analysed are logarithm of the mean fruit weight value (Lfw), the fruit length (Frl), the fruit diameter (Frd), the fruit shape (Frs), the flowering earliness(flw), Axis length (Axl), the number of leaves ( Nle ), the mean internode length ( Inl ), the mean internode growth time (Int), the axis growth speed (Axs). Coloured boxes indicate significant correlations ( $\mathrm{P}>0.05$ )

## iv. H3 X Vania population

Mean phenotypic values for each trait are listed in Table 6. Considering fruit traits, H3 produced narrow and elongated fruits with a thin flesh while fruits of Vania are large and with a heavier weight. However, contrarily to Yolo Wonder, Vania fruits are rather long with an oblate shape, resulting in a higher Frl/Frd ratio. Considering plant traits, flowering dates and internode length of Vania and H3 are very similar, but differences in the other traits are extreme with a much higher axis length and number of leaves, and a much more rapid stem and internode growth speed for H 3 than Vania.
The HV progeny displayed normal-like distributions for all the traits. Most of the F6 inbred lines displayed intermediate values between the parental lines for all the fruit traits, except pedicel length for which the average value of the progeny was higher than both parental lines and inbred F6 lines displayed transgressive phenotypes. Considering plant traits, the HV progeny displayed transgressive values for flowering earliness and internode length as a consequence of very similar parental values (Figure 4).
Heritability was high for each trait, ranging from 0.77 for the internode length to 0.97 for fruit weight (Table 6).
As observed in the YC and PY progenies, positive significant correlations ( $p<0.05$ ) were observed between fruit weight, length and diameter (pericarp thickness was not detected in this progeny). A late flowering date was associated with longer axis, higher number of leaves, and the speed of growth of the axis (and internode growth time) increased with the number of leaves (and axis length). Similarly to the PY progeny, significant negative correlations ( $p<0.05$ ) were found between fruit and plant traits, in particular, the fruit weight, length and diameter were found to decrease when the lateness of flowering, the axis length and the number of leaves increased. (Table 7).

QTLs analysis on horticultural traits


QTLs analysis on horticultural traits



Figure 4: Frequency distribution for the 11 traits analysed in the HV population: logarithm of the mean fruit weight value (Lfw), the fruit length (Frl), the fruit diameter (Frd), the fruit shape (Frs), the pedicel length (Pel), the flowering earliness(flw), Axis length (AxI), the number of leaves ( Nle ), the mean internode length ( Inl ), the mean internode growth time (Int) , the axis growth speed (Axs). Parental mean values are designated H3 (H3), Vania (Vania) while F1 indicates the hybrid mean.
QTLs analysis on horticultural traits

| Trait |  | Mean | Mean | Mean | Mean | Heritability |
| :--- | :--- | :---: | :---: | :---: | ---: | :---: |
|  |  | Vania | H3 | F1 | DH |  |
| Logarith of fruit weight | Lfw | 5.38 | 2.67 | 4.04 | 3.94 | 0.976 |
| Fruit length (mm) | Frl | 139.60 | 94.61 | 129.84 | 106.76 | 0.944 |
| Fruit diameter (mm) | Frd | 80.14 | 25.39 | 41.49 | 44.64 | 0.956 |
| Fruit shape | Frs | 1.74 | 3.73 | 3.13 | 2.45 | 0.94 |
| Peduncule length (mm) | Pel | 53.35 | 40.55 | 50.98 | 47.30 | 0.921 |
| Flowering (days) | Flw | 54.33 | 58.17 | 54.83 | 60.00 | 0.893 |
| Axis length (mm) | Axl | 17.1 | 45.7 | 31.3 | 29.67 | 0.964 |
| Number of leaves | Nle | 7.2 | 19.0 | 12.7 | 13.11 | 0.947 |
| Internode length (mm) | Inl | 2.4 | 2.4 | 2.5 | 2.30 | 0.773 |
| Mean internode growth time | Int | 5.6 | 2.3 | 3.2 | 3.40 | 0.894 |
| (days) |  |  |  |  |  |  |
| Axis growth speed |  | 3.6 | 10.6 | 7.9 | 6.56 | 0.94 |
| (mm/day) |  |  |  |  |  |  |

Table 6: Means and heritabilities of quantitative traits in the HV population for the parental, F1 and DH generations.

| QTLs analysis on horticultural traits |  |  |  |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Lfw | Frl | Frd | Frs | Pel | Flw | AxI | Nle | Inl | Int | Axs |
| Lfw |  | 0,37 | 0,89 | -0,51 | 0,29 | -0,08 | -0,55 | -0,42 | -0,31 | 0,50 | -0,62 |
| Frl |  |  | 0,12 | 0,54 | 0,08 | -0,32 | -0,32 | -0,34 | -0,02 | 0,29 | -0,26 |
| Frd |  |  |  | -0,74 | 0,34 | -0,05 | -0,51 | -0,41 | -0,24 | 0,49 | -0,59 |
| Frs |  |  |  |  | -0,22 | -0,21 | 0,19 | 0,11 | 0,18 | -0,22 | 0,31 |
| Pel |  |  |  |  |  | 0,15 | 0,15 | 0,13 | 0,06 | -0,07 | 0,10 |
| Flw |  |  |  |  |  |  | 0,55 | 0,62 | -0,04 | -0,30 | 0,26 |
| AxI |  |  |  |  |  |  |  | 0,82 | 0,40 | -0,72 | 0,94 |
| Nle |  |  |  |  |  |  |  |  | -0,17 | -0,91 | 0,73 |
| Inl |  |  |  |  |  |  |  |  |  | 0,21 | 0,46 |
| Int |  |  |  |  |  |  |  |  |  |  | -0,74 |
| Axs |  |  |  |  |  |  |  |  |  |  |  |
|  | $\begin{aligned} & \text { posi } \\ & \text { neg } \end{aligned}$ | and and |  |  |  |  |  |  |  |  |  | Table 7 Pearson correlation coefficient between traits measured in the HV progeny. Traits analysed are: logarithm of the

mean fruit weight value (Lfw), the fruit length (Frl), the fruit diameter (Frd), the fruit shape (Frs), the pedicel length (Pel), the
flowering earliness(flw), Axis length (Axl), the number of leaves (Nle), the mean internode length (Inl), the mean internode
growth time (Int), the axis growth speed (Axs). Coloured boxes indicate significant correlations ( $\mathrm{P}>0.05$ ).

## QTL detection

A list of the QTLs detected for all traits in the 3 progenies is presented in figure 6 together with alignment of genetic maps from the 3 crosses. Detailed data from each progeny are presented in the separate tables. For each trait and progeny, the QTLs were declared as significant when their LOD values overpassed the threshold value equivalent to $p=0.05$ after 1000 permutations (see material and methods). QTLs reported included those founded with both IM and CIM but also those found with only IM or CIM.

## Yolo Wonder X Criollo de Morelos population

Thirteen traits were analysed, and a total of 78 QTLs (Table 8) ranging from 2 to 12 per trait were identified. No significant digenic interactions were detectable ( $\mathrm{P}<10^{-6}$ ).

Logarithm of fruit weight
Seven QTLs were found, of which 5 detected with both methods, 1 with CIM and 1 with IM. Lfw3.1 and Lfw4.1 had the largest effect $\left(R^{2}=0.11\right.$ and 0.15 respectively) and all QTLs detected explained the $48 \%$ of the total phenotypic variation. For all QTLs, the alleles from the large fruited variety Yolo Wonder increased fruit weight.

| QTL |  | Marker | Direction | LOD | Variation explained |  | Heritability Additive |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  | Locus | Trait |  |  |
| Logarithm of fruit weight | Lfw3.1 | e40/m49_198y | Yolo Wonder | 5.14 | 0.11 | 0.48 | 0.96 | 0.12 |
|  | Lfw4.1 | p17/m32_240c | Yolo Wonder | 9.63 | 0.15 |  |  | 0.15 |
| Lod>3.08 | Lfw11.1 | e41/m61_137c | Yolo Wonder | 4.61 | 0.07 |  |  | 0.1 |
|  | Lfw12.1 CIM | e44/m51_263c | Yolo Wonder | 4.22 | 0.05 |  |  | 0.09 |
|  | LfwLG15.1 | e38/m60_224y | Yolo Wonder | 5.03 | 0.08 |  |  | 0.1 |
|  | LfwLG24.1 IM | e41/m54_184y | Yolo Wonder | 4.49 | 0.08 |  |  | 0.11 |
|  | LfwLG45.1 | Epms_402 | Yolo Wonder | 4.01 | 0.08 |  |  | 0.1 |
| Fruit length <br> Lod>3.12 | Frl3.1 | e36/m52_158 | Criollo de Morelos | 7.26 | 0.1 | 0.39 | 0.96 | -3.9 |
|  | Frl4.1 | e38/m60_109 | Yolo Wonder | 17.16 | 0.21 |  |  | 6.5 |
|  | Frl7.1 | e41/m61_140c | Yolo Wonder | 5.53 | 0.1 |  |  | 3.43 |
|  | FriLG22.1 | e34/m53_181c | Yolo Wonder | 5.99 | 0.1 |  |  | 3.95 |
| Fruit diameter <br> Lod $>3.15$ | Frd2.1 CIM | CD035 | Yolo Wonder | 4.73 | 0.12 | 0.91 | 0.97 | 2.23 |
|  | Frd3.1 | p14/m39_221y | Yolo Wonder | 3.72 | 0.11 |  |  | 2.17 |
|  | Frd4.1 | e41/m48_078 | Yolo Wonder | 4.63 | 0.05 |  |  | 1.46 |
|  | Frd8.1 CIM | p11/m49_274 | Yolo Wonder | 3.23 | 0.06 |  |  | 1.38 |
|  | Frd10.1 | e36/m47_145 | Yolo Wonder | 3.32 | 0.07 |  |  | 1.73 |
|  | Frd11.1 | e41/m61_137c | Yolo Wonder | 11.27 | 0.13 |  |  | 2.34 |
|  | Frd12.1 IM | e44/m51_263c | Yolo Wonder | 3.43 | 0.06 |  |  | 1.65 |
|  | FrdLG15.1 | e38/m60_224 | Yolo Wonder | 7.64 | 0.1 |  |  | 2.06 |
|  | FrdLG17.1 | e38/m61_144 | Yolo Wonder | 4.74 | 0.06 |  |  | 1.57 |
|  | FrdLG24.1 IM | e41/m54_184 | Yolo Wonder | 4.57 | 0.08 |  |  | 1.85 |
|  | FrdLG25.1 | p15/m40_091 | Yolo Wonder | 6.09 | 0.07 |  |  | 1.7 |
|  | FrdLG37.1 | p15/m40_319c | Yolo Wonder | 3.94 | 0.06 |  |  | 1.56 |
| Fruit shape <br> Lod $>3.12$ | Frs2.1 CIM | e36/m52_116 | Criollo de Morelos | 3.24 | 0.04 | 0.31 | 0.97 | -0.11 |
|  | Frs3.1 | e43/m54_256 | Criollo de Morelos | 11.75 | 0.15 |  |  | -0.22 |
|  | Frs4.1 | p14/m39_417 | Yolo Wonder | 4.01 | 0.05 |  |  | 0.11 |
|  | Frs4.2CIM | p25/m45_109 | Yolo Wonder | 3.83 | 0.08 |  |  | 0.16 |
|  | Frs10.1 | e38/m60_117 | Criollo de Morelos | 4.68 | 0.06 |  |  | -0.14 |
|  | Frs11.1 | e41/m61_270 | Criollo de Morelos | 4.41 | 0.07 |  |  | -0.13 |
|  | FrsLG17.1 | e40/m47_239 | Criollo de Morelos | 5.21 | 0.07 |  |  | -0.14 |
|  | FrsLG25.1 | e41/m54_351 | Criollo de Morelos | 3.79 | 0.06 |  |  | -0.12 |
| Pericarp thickness Lod>3.06 | Pet3.1 | PG101 | Yolo Wonder | 5.15 | 0.14 | 0.31 | 0.92 | 0.24 |
|  | Pet6.1 IM | p25/m45_185 | Yolo Wonder | 3.59 | 0.05 |  |  | 0.15 |
|  | Pet10.1 | e36/m47_145 | Yolo Wonder | 6.87 | 0.09 |  |  | 0.19 |
|  | Pet11.1 IM | e41/m61_270 | Yolo Wonder | 3.69 | 0.06 |  |  | 0.15 |
|  | Pet12.1CIM | e44/m51_263 | Yolo Wonder | 3.31 | 0.04 |  |  | 0.13 |
|  | PetLG15.1 | e38/m60_224y | Yolo Wonder | 7.82 | 0.1 |  |  | 0.2 |
|  | PetLG24.11M | e41/m54_184 | Yolo Wonder | 4.27 | 0.07 |  |  | 0.18 |
|  | PetLG27.1 | e40/m49 305 | Yolo Wonder | 5.75 | 0.08 |  |  | 0.18 |
| Number of loges <br> Lod>3.00 | NIo2.1 CIM | e36/m47_146 | Yolo Wonder | 3.42 | 0.05 |  |  | 0.08 |
|  | Nlo8.1 | Hpms1_214 | Yolo Wonder | 3.83 | 0.06 |  |  | 0.09 |
|  | Nlo12.1 | e44/m51_263c | Yolo Wonder | 4.08 | 0.05 |  |  | 0.08 |
|  | Nlo12.2 | e36/m47_237 | Yolo Wonder | 4.88 | 0.1 |  |  | 0.08 |
|  | NIoLG17.1 IM | e38/m61_144 | Yolo Wonder | 4.06 | 0.08 |  |  | 0.1 |

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|  | NIoLG22.1CIM | e34/m53_181c | Criollo de Morelos | 3.13 | 0.04 |  |  | -0.07 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | NIoLG25.1 | p15/m40_091 | Yolo Wonder | 8.12 | 0.13 |  |  | 0.12 |
|  | NIoLG30.1 | e44/m51_376 | Yolo Wonder | 8.12 | 0.11 |  |  | 0.12 |
|  | NIoLG39.1 | TntC07 | Yolo Wonder | 5.72 | 0.1 | 0.46 | 0.84 | 0.1 |
| Pedicel length | Pel1.1 CIM | e41/m61_199y | Yolo Wonder | 3.48 | 0.04 |  |  | 1.58 |
| Lod>3.03 | Pel2.1 | e36/m52_116 | Criollo de Morelos | 9.04 | 0.1 |  |  | -2.61 |
|  | Pel3.1 IM | e34/m53 077 | Criollo de Morelos | 5.48 | 0.12 |  |  | -2.79 |
|  | Pel4.1 | e41/m48_078 | Yolo Wonder | 5.68 | 0.07 |  |  | 2.05 |
|  | Pel12.1 | p25/m45_087 | Criollo de Morelos | 7.2 | 0.12 |  |  | -2.83 |
|  | PelLG42.1 | p25/m45_335 | Yolo Wonder | 6.14 | 0.08 | 0.37 | 0.95 | 2.15 |
| Flowering earliness | Flw1.1 | e34/M53_233c | Yolo Wonder | 8.04 | 0.1 |  |  | 0.72 |
| Lod>3.07 | Flw2.1 | Epms409 | Criollo de Morelos | 6.64 | 0.1 |  |  | -0.7 |
|  | Flw4.1 CIM | e38/m61_168 | Yolo Wonder | 3.34 | 0.05 |  |  | 0.49 |
|  | FlwLG15.1 | e38/m60_224 | Yolo Wonder | 4.83 | 0.07 |  |  | 0.58 |
|  | FlwLG17.1 | p14/m33_851 | Yolo Wonder | 4.19 | 0.07 | 0.15 | 0.88 | 0.59 |
| Axis length | Axl2.1 | e36/m47_146 | Criollo de Morelos | 4.65 | 0.07 |  |  | -0.96 |
| Lod>3.02 | AxI6.1 IM | p14/m41_060y | Yolo Wonder | 3.29 | 0.05 |  |  | 0.83 |
|  | AxI9.1 | e37/m54_92 | Criollo de Morelos | 3.83 | 0.08 |  |  | -0.98 |
|  | AxILG24.1 IM | Epms376 | Yolo Wonder | 3.53 | 0.06 |  |  | 0.88 |
|  | AxILG47.1 | p14/m33_311 | Yolo Wonder | 6.15 | 0.1 | 0.42 | 0.94 | 1.18 |
| Number of leaves | Nle3.1 IM | e40/m49_198y | Criollo de Morelos | 3.29 | 0.06 |  |  | -0.36 |
| Lod>3.17 | $\begin{array}{\|l} \hline \text { NleLG38.1 } \\ \text { CIM } \\ \hline \end{array}$ | p17/m32_344 | Criollo de Morelos | 3.3 | 0.04 |  |  | -0.31 |
|  | NIeLG45.1 IM | Epms_402 | Criollo de Morelos | 3.08 | 0.05 |  |  | -0.33 |
|  | NIeLG47.1 | p14/m33_311c | Yolo Wonder | 6.31 | 0.11 | 0.25 | 0.92 | 0.49 |
| Internode length | Inl1.1 | e36/m52_190y | Yolo Wonder | 4.22 | 0.06 |  |  | 0.07 |
| Lod>3.13 | Inl2.1 | e36/m47_146 | Criollo de Morelos | 6.25 | 0.11 |  |  | -0.08 |
|  | InILG28.1 IM | e41/m54_221c | Yolo Wonder | 5.1 | 0.08 | 0.1 | 0.91 | 0.08 |
| Internode growth time | Int4.1IM | e42/M48_116y | Yolo Wonder | 3.47 | 0.05 |  |  | 0.1 |
| Lod>3.00 | IntLG47.1 | p14/m33_311 | Criollo de Morelos | 4.86 | 0.11 | 0.07 | 0.9 | -0.14 |
| Axis growth speed | Axs2.1 CIM | e36/m47_146 | Criollo de Morelos | 6.27 | 0.1 |  |  | -0.26 |
| Lod>3.05 | Axs2.2 CIM | p25/m42_268 | Yolo Wonder | 3.69 | 0.05 |  |  | 0.18 |
|  | Axs4.1 | e38/m61_168 | Criollo de Morelos | 4.48 | 0.07 |  |  | -0.2 |
|  | Axs9.1 | CT145 | Criollo de Morelos | 4.46 | 0.09 |  |  | -0.24 |
|  | AxsLG32.1 | p15/m43_158 | Yolo Wonder | 5.08 | 0.05 | 0.44 | 0.93 | 0.25 |

Table 8: List of QTLs detected in the F6 progeny. Marker indicates the closest marker to te QTL, direction indicates the parent which contributes to increase the numeric value of the trait. LOD values indicates the threshold for declaring the presence of a QTL for each trait at $p>0.05$. IM and CIM stand for Interval mapping and Composite interval mapping respectively.

## Fruit length

Four QTLs were detected with both methods. Frl4.1 displays the major effect $\left(R^{2}=0.21\right)$ with the favourable allele from Yolo Wonder. The total of QTLs found explained $38 \%$ of the total phenotypic variation. Both parental alleles contributed for the length of fruits: the Criollo de Morelos allele for Frl3.1 and Yolo Wonder alleles for Frl4.1, Frl7.1 and FriLG22.1.

## Fruit diameter

Twelve QTLs were found for this traits, of which 8 detected with both methods, 2 with IM and 2 with CIM. Frd11.1, Frd3.1 and Frd2.1CIM are the ones with most significant effects $\left(R^{2}=0.13,0,12\right.$ and 0,11 respectively) while the total phenotypic variability explained by all QTLs is equal to $91 \%$. At all the QTLs the Yolo Wonder alleles contributed to increase the diameter of fruits.

## Fruit shape

Eight QTLS were detected, of which 2 with only CIM. Frs3.1 is the most important QTL with a $\mathrm{R}^{2}=0.15$. Both parental alleles contributed to the shape of the fruit (increasing the ratio Frl/Frd) with 6 alleles from Criollo de Morelos and two from Yolo Wonder. The total phenotypic variation explained by QTLs was $31 \%$.

## Pericarp thickness

Eight QTLs were found ( 4 with IM and CIM, 3 with only IM and 1 with CIM). Pet3.1 and PetLG15.1 were the mostly important with a $R^{2}=0.14$ and 0,10 respectively. Total phenotypic variation explained by QTLs was $31 \%$ and only Yolo Wonder alleles contributed to the trait.

Number of locules
Nine QTLS ( 6 for both methods, 1 with IM and 2 with CIM) were detected. NoLG25 and NoLG30 were the most important with a $\mathrm{R}^{2}=$
0.13 and 0,11 respectively. Only Yolo Wonder alleles increased the number of locules and total variability explained by QTLs was $46 \%$.

## Pedicel length

Six QTLs ( 4 with CIM and IM, 1 with IM and 1 with CIM) were found. Pel3.1Im and Pel12.1 are the most significant QTLs ( $\mathrm{R}^{2}=0.12$ for both QTLs). Both parental alleles contributed to increase the pedicel length (Pel2.1, Pel3.1IM and Pel12.1 for Criollo de Morelos and Pel1.1CIM, Pel4.1 and PelLG42 for Yolo Wonder). Total phenotypic variation explained was equal to $37 \%$.

Flowering earliness
Five QTLs (of which one detected with only CIM) were found and Flw1.1 and Flw2.1 are the most important $\left(R^{2}=0.10\right)$. Both parental alleles contributed to increase the trait and total phenotypic variation explained was $15 \%$.

Axis length
Five QTLs ( 3 in common for $I M$ and CIM and 2 found with IM) were found. AxILG47.1 is the most important QTL with $R^{2}=0.1$. Both parental alleles contributed to increase the axis length and total phenotypic variability explained was $42 \%$.

## Number of leaves

Four QTLs of which one detected with IM and CIM, 2 with IM and 1 with CIM were found. NleLG47.1 is the one with strongest effect $\left(R^{2}=0.11\right)$. The total phenotypic variation explained was $25 \%$ and both parental alleles contributed to increase the trait (Criollo de Morelos for Nle 3.1 IM , Ne38.1CIm and NleLG45.1IM and Yolo Wonder for NleLG47.1).

Internode length
Three QTLs were detected and Intl2.1 was the most important with $\mathrm{R}^{2}=$ 0.11 . Criollo de Morelos allele increased the trait for $\operatorname{In} 2.1$ while Yolo Wonder alleles for $\operatorname{In} 11.1$ and $\operatorname{In} \mid 28.1 \mathrm{IM}$. Toal phenotypic variability explained was only $10 \%$.

Internode growth time
Only 2 QTLs were detected (1 with both methods and 1 with IM). IntLG47.1 was the most significant $\left(R^{2}=0.11\right)$. Yolo Wonder allele contributed for $\operatorname{Int} 4.1 \mathrm{IM}$ while Criollo de Morelos alleles contributed for IntLG47.1 Total phenotypic variability explained was $7 \%$.

Axis growth speed
Five QTLs (2 with IM and CIM, 3 with CIM) were found. Axs2.1CIM showed the highest effect with $R^{2}=0.1$. Total phenotypic variation explained by QTLs is $44 \%$. Criollo de Morelos alleles contributed for Axs2.1CIM, Axs4.1 and Axs9.1 while Yolo Wonder alleles influenced Axs2.2CIM and AxsLG32.

## Perennial X Yolo Wonder population

Ten traits were analysed in this progeny, and a total of 28 QTLs (Table 9), ranging from 1 to 5 per trait were identified. No significant digenic interactions were detectable for all the traits in study $\left(\mathrm{P}<10^{-6}\right)$.

Logarithm of fruit weight
Five QTLs were found, of which 3 detected with both methods and 2 with only IM. Lfw2.1IM, Lfw7.1IM and Lfw11.1IM had the largest effect $\left(R^{2}=0.21,0.23\right.$ and 0.22 respectively) while all detected QTLs detected explain $57 \%$ of the total phenotypic variation. For all QTLs, the alleles from variety Yolo Wonder were found to increase fruit weight.

## Fruit length

A total of 4 QTLs were detected, of which two with CIM method. Fri3.1 was the one with major effect $\left(R^{2}=0.26\right)$ while on the whole the QTLs found explained $49 \%$ of the total phenotypic variation. Both parental alleles contributed to increase the fruit length i.e.: the Perennial allele for Frl3.1 and Yolo Wonder alleles for Frl2.1 Frl3.2CIM and Frl11.1CIM.

## Fruit diameter

Five QTLs were found for this trait, 3 detected with both methods, and 2 with IM. Frd3.1, Frd7.1IM and Frd2.1 are the ones with most remarkable effects ( $R^{2}=0.22,0.19$ and 0.18 respectively) while the total phenotypic variability explained by QTLs was $67 \%$. Only the alleles from Yolo Wonder parent increased the fruit diameter.

## Fruit shape

The total phenotypic variation explained by QTLs was $56 \%$, although only 2 QTLs were found with both IM and CIM. Frs3.1 is the most influent ( $R^{2}=0.64$ ). Only Perennial alleles contributed to the shape of the fruit.

Flowering earliness
Two QTLs (of which one with only CIM) were detected with Flw2.1 showing the greatest effect ( $R^{2}=0.67$ ). Only Perennial parent's alleles increased the trait value and total phenotypic variation explained was 56\%.

Axis length
One QTL was detected with both methods on chromosome 2. The $\mathrm{R}^{2}$ value is equal to $0.39 \%$ and Perennial allele increased the trait value.

## Number of leaves

Three QTLS of which one detected with IM and CIM, and 2 with CIM were found. Nle 2.1 is the one with strongest effect $\left(\mathrm{R}^{2}=0.37\right)$. The total phenotypic variation explained is $49 \%$ and both parental alleles contributed to the trait (Perennial for Nle 2.1 and $\mathrm{Nle5.1CIM}$, and Yolo Wonder for Nle12.1CIM).

Internode length
2 QTLs were detected and Inl2.2 was the most important with $R^{2}=0.23$. Perennial alleles increased the trait valu for both QTLs. Total phenotypic variability explained was $22 \%$.

Internode growth time
Three QTLs were detected ( 2 with both methods and 1 with CIM). Int2.1 was the most significant ( $R^{2}=0.22$ ). Yolo Wonder allele increased the trait value for $\operatorname{Int2} 21$ and $\operatorname{Int5} 5$.1CIM while Perennial alleles increased for Int12.1 Total phenotypic variability explained was $0.37 \%$.

Axis growth speed
A single QTL (Axs2.1) were found with a $R^{2}=0.46$. Perennial allele increased the trait value detected.


Table 9: QTLs detected in the PY population. Marker indicates the closest marker to te QTL, direction indicates the parent which contributes to increase the numeric value of the trait. LOD values indicates the threshold for declaring the presence of a QTL for each trait at $\mathrm{p}>0.05$. IM and CIM stand for Interval mapping and Composite interval mapping respectively

## H3 X Vania population

Eleven traits were analysed in this progeny and a total of 33 QTLs (Table 10), ranging from 1 to 5 per trait were identified. No significant digenic interactions were detectable for all the traits in study $\left(\mathrm{P}<10^{-6}\right)$.

Logarithm of fruit weight
Four QTLs were found, all detected with both methods. Lfw11.1, Lfw 11.2 and Lfw2.1 had the largest effect ( $R^{2}=0.28,0.26$ and 0.24 respectively) and all QTLs detected explain the $47 \%$ of the total phenotypic variation. For all QTLs, the alleles from the large fruited accession Vania increased fruit weight.

Fruit length
Five QTLs were detected, of which one with both methods, 3 using CIM and one by IM. Frl9.2 was the one with major effect $\left(R^{2}=0.15\right)$ and the total of QTLs found explained the 29\% of the total phenotypic variation. Both parent's allele increased for the length of fruits: the H3 allele for Frl1.1CIM and Vania alleles for Frl2.1CIM, Frl7.1CIM, Frl9.1CIM and Frl9.2.

Fruit diameter
Five QTLs were found for this trait, 2 detected with both methods, 1 with IM and 2 with CIM. Frd11.1 and Frd2.1IM are the ones with most significant effects ( $\mathrm{R}^{2}=0.26$ and 0.22 respectively) while the total phenotypic variability explained by all QTLs was equal to $58 \%$. Only the Vania parental alleles contributed to increase the diameter of fruits.

## Fruit shape

Four QTLs were detected, of which 2 with CIM. Frs11.1 is the most important QTL with a $\mathrm{R}^{2}=0.19$. Only H 3 alleles contributed to increase the ratio of length to width of the fruit and the total phenotypic variation explained by all the QTLs is $48 \%$.

Pedicel length
Four QTLs ( 3 with CIM and IM and 1 with CIM) were found. Pel8.1 is the most significant $\left(R^{2}=0.27\right)$. Both parents contributed to increase the pedicel length (H3 for Pel6.1, Vania for Pel8.1, Pel11.1 and Pel11.2). Total phenotypic variation explained is equal to $52 \%$.

Flowering earliness
Two QTLs were detected with both methods and Flw2.1 is the one with major effect ( $R^{2}=0.21$ ). Both parental alleles increased the flowering date and total phenotypic variation explained was $17 \%$

## Axis length

Two QTLs were found by CIM and IM. AxI2.1 is the most important QTL with $R^{2}=0.36$. Only the H 3 parent alleles increased the axis length. Total phenotypic variability explained was $37 \%$.

## Number of leaves

Only one QTL was detected with both methods. Nle2.1 has a strong effect ( $R^{2}=0.47$ ). The total phenotypic variation explained was $47 \%$ and the H 3 allele contributed to the trait.

## Internode length

Two QTLs were detected (one by IM and one by both methods). Inl1.1 was the most important with $R^{2}=0.22$. H 3 alleles increased the
internode length at both QTLs and the total phenotypic variability explained was $27 \%$.

Internode growth time
One QTL detected by both CIM and IM was found. Int2.1 has a strong effect with $R^{2}=0.51$. H3 allele increased the growth speed of the main axis at the QTL detected.

## Axis growth speed

Three QTLs were detected with both methods. Axs 2.1 was the most significant $\left(R^{2}=0.21\right)$. H3 alleles contributed for all the QTLs detected. Total phenotypic variability explained was $0.43 \%$.

|  | QTL | Marker | Direction | LOD | Variation explained |  | Heritability | Additive |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  | Locus | Trait |  |  |
| Logarithm of fruit weight | Lfw2.1 | e40/m48_229v | Vania | 3.83 | 0.24 |  |  | 0.34 |
|  | Lfw3.1 | PG101 | Vania | 3.26 | 0.15 |  |  | 0.27 |
| Lod>3.01 | Lfw11.1 | B10_0.9 | Vania | 6.60 | 0.28 |  |  | 0.38 |
|  | Lfw11.2 | CT101 | Vania | 9.67 | 0.26 | 0.47 | 0.97 | 0.36 |
| Fruit length <br> Lod>3.04 | Frl1.1CIM | TG370 | H3 | 3.26 | 0.1 |  |  | -8.93 |
|  | Frl2.1CIM | TLL080 | Vania | 3.15 | 0.13 |  |  | 10.42 |
|  | Fri7.1CIM | C00088 | Vania | 3.86 | 0.12 |  |  | 10 |
|  | Frl9.1 IM | P09_0.25 | Vania | 2.84 | 0.12 |  |  | 10.02 |
|  | Fri9.2 | Ae11. 1.3 | Vania | 4.66 | 0.15 | 0.29 | 0.94 | 11.13 |
| Fruit diameter <br> Lod>2.87 | Frd2.1 IM | e40/m48_229v | Vania | 3.62 | 0.22 |  |  | 7.06 |
|  | Frd3.1 | 007_075 | Vania | 4.49 | 0.11 |  |  | 4.9 |
|  | Frd8.1CIM | TG017 | Vania | 3.50 | 0.08 |  |  | 4.25 |
|  | Frd10.1CIM | e40/m48_227v | Vania | 3.49 | 0.08 |  |  | 4.25 |
|  | Frd11.1 | TG104 | Vania | 9.98 | 0.26 | 0.58 | 0.95 | 7.99 |
| Fruit shape <br> Lod>2.98 | Frs1.1CIM | P13/m47_361h | H3 | 3.24 | 0.1 |  |  | -0.3 |
|  | Frs3.1 | D11_0.6h | H3 | 4.65 | 0.15 |  |  | -0.38 |
|  | Frs10.1CIM | e40m49_227v | H3 | 3.01 | 0.08 |  |  | -0.28 |
|  | Frs11.1 | B10_09 | H3 | 5.21 | 0.19 | 0.48 | 0.94 | -0.42 |
| Peduncule length <br> Lod>3.06 | Pel6.1CIM | e36/m59 380h | H3 | 4.89 | 0.13 |  |  | -6.1 |
|  | Pel8.1 | e36/m49_125h | Vania | 9.87 | 0.27 |  |  | 8.56 |

QTLs analysis on horticultural traits

|  | Pel11.1 | CT101 | Vania | 4.20 | 0.11 | 0.52 | 0.92 | 5.7 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Pel11.2 | C02_05 | Vania | 4.19 | 0.13 |  |  | 6.14 |
| Flowering earliness | Flw2.1 | TLL080 | H3 | 5.24 | 0.21 | 0.17 | 0.89 | -3.84 |
| Lod>2.85 | Flw8.1 | e32/m49_203v | Vania | 4.29 | 0.14 |  |  | 3.08 |
| Axis length | Axl2.1 | e40/m48_229v | H3 | 11.03 | 0.36 | 0.37 | 0.96 | -9.3 |
| Lod>2.79 | AxI11.1 | AE11_1.6v | H3 | 5.64 | 0.14 |  |  | -6 |
| Number of leaves | Nlw2.1 | e40/m48_229v | H3 | 14.47 | 0.47 | 0.47 | 0.95 | -4 |
| Lod>2.99 |  |  |  |  |  |  |  |  |
| Internode length | Inl1.11M | e40/m47_077h | H3 | 4.12 | 0.22 | 0.27 | 0.77 | -0.32 |
| Lod>2.96 | Inl11.1 | B10_09 | H3 | 5.02 | 0.14 |  |  | -0.26 |
| Internode growth time | Int2.1 | TLL080 | Vania | 14.05 | 0.51 | 0.51 | 0.89 | 1.1 |
| Lod>3.03 |  |  |  |  |  |  |  |  |
| Axis growth speed Lod $>2.84$ | Axs2.1 | TII080 | H3 | 5.18 | 0.21 | 0.43 | 0.94 | -0.15 |
|  | Axs6.1 | CMI380h | H3 | 3.37 | 0.11 |  |  | -0.11 |
|  | Axs 11.1 | AE11_1.6 | H3 | 3.53 | 0.12 |  |  | -0.11 |

Table 10: QTLs detected in the HV population. Marker indicates the closest marker to te QTL, direction indicates the parent which contributes to increase the numeric value of the trait. LOD values indicates the threshold for declaring the presence of a QTL for each trait at $p>0.05$. IM and CIM stand for Interval mapping and Composite interval mapping respectively

## QTLs detection in the subpopulations selected from the recombinant inbred progeny YC

In order to compare the performance of QTL detection in reduced populations obtained by selective sampling, QTL analysis was performed in the 2 sub-populations $A$ (141 inbred lines) and $B$ (93 inbred lines) that were sampled by means of MapPop software (Brown and Vision 2000) from the recombinant inbred lines from the YC population (297 inbred lines). Only three traits were submitted to the analysis: one fruit trait (Lfw) that was controlled by QTIs with variable $R^{2}$ values ( 0.15 to 0.08 ), one plant trait (AxI) controlled by QTLs with low $R^{2}$ values (0.10 to 0.6), and one disease resistance trait that was shown to be controlled by a high effect QTL $\left(R^{2}=0.53\right)$ and minor QTLs $\left(R^{2}\right.$ from 0.12 to 0.03$)$ (Table 11).

Logarithm of fruit weight
Of the 7 QTLs detected with 297 individuals, only 3 (Lfw3.1, Lfw4.1 and LfwLG15.1) were found using the subpopulations of 141 plants. Lfw3.1 is the most important with a $R^{2}=0.18$ while total phenotypic variation explained was $30 \%$. Only Yolo Wonder alleles contributed to the trait. 3 QTLs were also found using 93 individuals (Lfw4.1IM, Lfw11.1IM and LfwLG15.1) with LfwLg15.1being the most important with a $R^{2}=0.18$. Total phenotypic variability explained by those QTLs was $23 \%$.

## Axis Length

Of the 5 QTLs previously found using 297, no QTLs were detected using 141 individuals. Surprisingly using 93 individuals one QTL on chromosome 12 was found with $R^{2}=0.2$.

## Receptivity

Using the original population, one QTLs was found on chromosome P5. The use of the subpopulation composed of 141 individuals allowed to detect the same QTL with a $R^{2}=0.43$. By using the subpopulation of 93 individuals we found the same QTL with a $\mathrm{R}^{2}=0.35$. Only the resistant Criollo de Morelos alleles increased the resistance.

| Trait/ | RIL YC (n=297) |  |  |  | Subpop.A (n=141) |  |  |  | Subpop.B (n=93) |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | Pos. | LOD | $\begin{gathered} \text { R2- } \\ \text { Locus } \end{gathered}$ | R2trait | Pos. | LOD | $\begin{gathered} \text { R2- } \\ \text { Locus } \end{gathered}$ | R2- <br> trait | Pos. | LOD | $\begin{gathered} \text { R2- } \\ \text { Locus } \end{gathered}$ | R2- <br> trait |
| LfW |  |  |  |  |  |  |  |  |  |  |  |  |
| Lfw3.1 | $\begin{array}{\|l\|} \hline \mathrm{e} 40 / \mathrm{m} 4 \\ 9 \_198 \mathrm{y} \\ \hline \end{array}$ | 5.14 | 0.11 |  | $\begin{aligned} & \hline \text { e40/m4 } \\ & 9 \_198 y \\ & \hline \end{aligned}$ | 7.22 | 0.1813 |  |  |  |  |  |
| Lfw4.1 | $\begin{array}{\|l\|} \hline \mathrm{p} 17 / \mathrm{m} 3 \\ 2 \_240 \mathrm{c} \\ \hline \end{array}$ | 9.63 | 0.15 |  | $\begin{aligned} & \mathrm{p} 17 / \mathrm{m} 3 \\ & 2 \_240 \mathrm{c} \\ & \hline \end{aligned}$ | 4.06 | 0.1014 |  | $\begin{aligned} & \mathrm{p} 17 / \mathrm{m} 3 \\ & 2 \_240 \mathrm{c} \\ & \hline \end{aligned}$ | 3.97 | 0.12 |  |
| Lfw11.1 | $\begin{array}{\|l\|} \hline \mathrm{e} 41 / \mathrm{m} 6 \\ 1 \_137 \mathrm{c} \\ \hline \end{array}$ | 4.61 | 0.07 |  |  |  |  |  | $\begin{aligned} & \mathrm{P} 25 / \mathrm{m} 4 \\ & 5 \_274 \mathrm{y} \\ & \hline \end{aligned}$ | 4.2 | 0.14 |  |
| $\begin{array}{\|l\|} \hline \text { Lfw12.1 } \\ \text { CIM } \\ \hline \end{array}$ | $\begin{array}{\|l\|} \hline \mathrm{e} 44 / \mathrm{m} 5 \\ 1 \_263 \mathrm{c} \\ \hline \end{array}$ | 4.22 | 0.05 |  |  |  |  |  |  |  |  |  |
| $\begin{array}{\|l\|} \hline \text { LfwLG1 } \\ 5.1 \\ \hline \end{array}$ | $\begin{aligned} & \mathrm{e} 38 / \mathrm{m6} \\ & 0 \quad 224 \mathrm{y} \\ & \hline \end{aligned}$ | 5.03 | 0.08 |  | $\begin{aligned} & \hline \mathrm{e} 38 / \mathrm{m6} \\ & 0 \_224 \mathrm{y} \\ & \hline \end{aligned}$ | 4.7 | 0.12 |  | $\begin{array}{\|l\|} \hline \mathrm{e} 38 / \mathrm{m6} \\ 0 \quad 224 \mathrm{y} \\ \hline \end{array}$ | 5.23 | 0.1964 |  |
| $\begin{aligned} & \text { LfwLG2 } \\ & 4.1 \mathrm{IM} \\ & \hline \end{aligned}$ | $\begin{array}{\|l} \hline \mathrm{e} 41 / \mathrm{m} 5 \\ 4 \_184 \mathrm{y} \\ \hline \end{array}$ | 4.49 | 0.08 |  |  |  |  |  |  |  |  |  |
| LfwLG4 5.1 | $\begin{aligned} & \text { Epms_4 } \\ & 02 \end{aligned}$ | 4.01 | 0.08 | 0.48 |  |  |  | 0.30 |  |  |  | 0.23 |
| Ax\| |  |  |  |  |  |  |  |  |  |  |  |  |
| Axl2.1 | $\begin{array}{\|l\|} \hline \text { e36/m4 } \\ \hline 7 \_146 \\ \hline \end{array}$ | 4.65 | 0.07 |  |  |  |  |  |  |  |  |  |
| AxI6.1 <br> IM | $\begin{aligned} & \hline \mathrm{p} 14 / \mathrm{m4} \\ & 1 \_060 \mathrm{y} \\ & \hline \end{aligned}$ | 3.29 | 0.05 |  |  |  |  |  |  |  |  |  |
| Ax19.1 | $\begin{aligned} & \text { e37/m5 } \\ & 4 \_92 \\ & \hline \end{aligned}$ | 3.83 | 0.08 |  |  |  |  |  |  |  |  |  |
| AxI12.1 |  |  |  |  |  |  |  |  | $\begin{aligned} & \text { MPG00 } \\ & 4 \end{aligned}$ | 5.03 | 0.205 |  |
| $\begin{array}{\|l} \hline \text { AxILG24 } \\ .1 \mathrm{IM} \\ \hline \end{array}$ | $\begin{aligned} & \text { Epms37 } \\ & 6 \end{aligned}$ | 3.53 | 0.06 |  |  |  |  |  |  |  |  |  |
| $\begin{array}{\|l\|} \hline \text { AxILG47 } \\ .1 \\ \hline \end{array}$ | $\begin{array}{\|l\|l\|l\|} \hline \text { p14/m3 } \\ 3 \_311 \\ \hline \end{array}$ | 6.15 | 0.1 | 0.42 |  |  |  |  |  |  |  | 0.2 |
| REC |  |  |  |  |  |  |  |  |  |  |  |  |
| Pc5.1 | $\begin{array}{\|l\|} \hline \mathrm{e} 42 / \mathrm{m} 4 \\ 8 \_155 \mathrm{y} \\ \hline \end{array}$ | 33.8 | 0.53 | 0.53 | $\begin{array}{\|l\|l\|} \hline \mathrm{e} 42 / \mathrm{m} 4 \\ 8 \_155 \mathrm{y} \\ \hline \end{array}$ | 15.84 | 0.43 | 0.43 | $\begin{array}{\|l\|} \hline \mathrm{e} 42 / \mathrm{m} 4 \\ 8 \_155 \mathrm{y} \\ \hline \end{array}$ | 10.85 | 0.34 | 0.34 |

Table 11: QTL detection in the original and reduced populations obtained by selective sampling.

## Discussion

Aim of this part of the experimental work was to detect QTLs controlling horticultural traits in the intra-specific genetic linkage map (YC) based on RILs previously developed (see chapter 2), and to validate their position by alignment with other two intra-specific genetic linkage maps (PY and HV) based on DH progenies published by Lefebvre et al. (2002). This will make it possible to obtain more precise and reliable information on the genetics of horticultural traits and identify genomic regions playing a key role in controlling their expression.

Distribution of phenotypic data between the three populations were similar for almost all of the traits. An exception was the distribution of the length of the fruit in the HV population, presumably due to the more elongated shape on the barriers of the parent Vania in comparison with the parent Yolo Wonder.

Correlations within fruit and plant traits were always highly significant. Correlations between plant and fruit traits were not detected in the YC progeny, but were significant in the PY and HV populations. A possible explanation might be found out in the genetic structure of the latter two populations. Indeed, the DH progenies were originated by a lower number of recombinant events and presumably they might have conserved larger association blocks which were responsible for the detected correlations. Classical quantitative genetics assumes that trait correlation can be attributed to the effect of pleiotropy or to the tight linkage of genes. From the present results it appears that pleiotropy might be the major reason for the observed correlations between plant and fruit traits in PY and HV populations, in which several QTLs responsible for them co-localised in the same genomic regions (i.e. on
chromosome P2 and P11). On the contrary, in the YC population few QTLs for plant and fruit traits co-localised, and this might justify the absence of significant correlations. High reproducibility of the data between repeats allowed to estimate the heritability for the traits in study, although phenotypic tests were not performed in different years and environments. The $h^{2}$ values were very high for all traits and in all the populations, and comparable to those detected by Ben Chaim et al. (2001a) but higher than those found by Rao et al. (2003).
Only QTLs detected with IM and/or CIM and above the LOD threshold at $\mathrm{p}=0.05$ were considered. Some major effect QTLs were found in all the populations: i.e. Frl4.1 and Frs3.1 in the YC, Frl3.1, Frs3.1 and Nle2.1 in the PY and Axl2.1 and Int2.1 in the HV. The percentage of genetic variation explained by QTLs was highly variable in relation to the trait and population considered. Fruit diameter always showed the highest $\mathrm{R}^{2}$ value (from 0.91 in YC to 0.58 in HV). On the contrary the internode lenght and the internode growth time showed, on average, the lowest $R^{2}$ values. Moreover, the highest percentage of explained variation was found when few QTLs with high individual $R^{2}$ (e.g. fruit shape or flowering earliness in PY population) or many QTLs with minor effects (e.g. fruit diameter in all progenies) were detected. Global $\mathrm{R}^{2}$ may depend on the accuracy of the linkage map, the between-marker distances and the mapping algorithm used (Jansen et al. 1995). In addition, the genetic structure of the trait, the dimension of the segregating populations (Melchinger et al. 1998, Utz et al. 2000), genetic background, environment, and interactions among QTL, might bring to an uncomplete QTL detection. Indeed, the high level of heritabilities detected and the incomplete percentage of variation explained by QTLs for all traits in all the progenies, lead to assume that not all QTLs were detected. Moreover the genome coverage of the YC map was about $85 \%$ which presumably limited the power of detecting low effect QTLs.

## Effect of selective sampling and reduced population size on QTL detection

An optimised sampling method approach based on the use of MapPop software (Brown and Vision 2000) was applied to evaluate the reliability of QTL analysis in reduced subpopulations of 141 and 93 RILs.

As expected the number of detectable QTLs decreased by reducing population size. Only QTLs with the highest LOD were found for fruit weight and receptivity. For axis length, none of the previously spotted QTLs was identified in the sub-populations but a new one was identified on chromosome P12 in the population of 93 RILs. The only explication for that seems to be an error due to the reduced number of individuals assayed. The $R^{2}$ for each trait was checked in the 2 sub-populations and decreased with the decrease in the number of individuals tested. On the basis of the results previously reported (see chapter 2) selective genotyping is suitable for the identification of new markers to be added in a previously developed genetic map. On the contrary, the selective genotyping approach seems not appropriate for QTL detection.

Genetic architecture of the QTLs of the different traits
v. Allelic diversity, cluster organization and conservation of QTLs in pepper germplasm

Most of the chromosomes were involved in the genetic control of at least one of the 13 traits analysed, and the organization of QTLs in clusters explains the correlations found between traits.

QTLs controlling fruit traits were mainly concentrated on P2, P3, P10 and P11 in all the progenies, together with progeny specific clusters on P4 and P12 for YC. Clusters of QTLs for fruit traits on P2, P3, P4, P10 were previously detected in intra as well as inter-specific crosses with
major effects on P3 (Ben Chaim et al. 2001a, Rao et al. 2003). A new cluster on P11 with major effects on fruit weight and diameter was detected in our 3 populations. The same cluster with minor effects was also detected in the inter-specific cross by Rao et al. (2003).
The comparative analysis of QTLs in the intra-specific pepper populations showed coherence in the results, thus allowing QTLs positions in the pepper genome to be confirmed.
In the three populations (Table 12) alleles from both parents were found to contribute for fruit length and pedicel length.
Major effect QTLs controlling fruit traits were found to co-localize on chromosome P2 for fruit diameter, on chromosomes P3 for fruit shape and fruit diameter and on chromosome P11 for fruit diameter. The P2, P3 and P11 alleles increasing the fruit diameter all originated from large the fruited parents, while the P3 and P10 alleles controlling the fruit shape always derived from the small fruited ones. Selection for elongated shape may require to select for the alleles of small fruited varieties at major QTLs in chromosome P3 and P10, but length of the fruits can be increased in selecting alleles from large fruited varieties at QTLs in Chromosomes P2, P4, P7 and P11.
Concerning plant traits, major effect QTLs were always found to cluster on P2 for flowering date, axis length and number of leaves, thus showing a tight co-localization. The P2 alleles increasing the axis length, the flowering earliness and the number of leaves originated from small fruited parents while those increasing the axis speed growth from large fruited ones. Those traits (including the calculated parameters Axs, Inl, Int) were presumably under a common genetic control (pleiotropy) due to their high level of co-localization and correlation.
By considering both plant and fruit traits, putative pleitropic control was detected on chromosome P2. QTLs for fruit weight, fruit length and internode growth time are influenced by large fruited parent alleles in the HV and PY populations; in addition the fruit diameter appeared
controlled by large fruited parents in all the three progenies. Vice versa flowering earliness, axis length, internode length and axis growth speed were always controlled by small fruited parent. These results show that, as previously observed in tomato (Bernacchi et al. 1998), chromosome P2 and T2 deeply influence the fruit size in both species.

The QTLs in common between the two or three populations analysed are listed in table 13. Plant traits are in general controlled by both parental alleles in the YC population, while in the PY and HV populations only two (number of leaves, internode growth time) and one (flowering earliness) traits respectively are controlled by both parental alleles, despite the presence of transgressive individuals for many traits. These results presumably might be explain by the presence of undetected QTLs.

QTLs analysis revealed the presence for the YC, PY and HV populations of several QTLs on chromosome P2. In PY and HV these QTLs are located in the top part of the chromosome while, in the YC they are located in the middle part of chromosome P2. A possible explanations is the presence in the top part of the chromosome of more common mapped markers in two DH populations.
Transgressive segregation occurred for several traits. in particular for plant traits. Several authors (DeVicente and Tanksley, 1993, Rieseberg et al. 1999, Rieseberg et al. 2003) pointed out that transgressive segregation might result from the appearance of combinations of alleles from both parents that have effect on the same direction. Moreover. DeVicente and Tanksley (1993) stated that the more similar the phenotype of the parents is, the more likely that transgressive individuals would occur, as we observed for pedicel length and the number of leaves in the YC population, for flowering earliness, axis length, internode length internode growth time and axis growth speed in the PY population and for flowering earliness and internode length in the HV population. To confirmation of that, in the YC population no
transgressive segregation was observed for the traits affected by only Yolo Wonder alleles as no one of the YC individual presented a phenotypic value similar to large fruited parent. On the contrary in the PY population, trasgressive segregation was observed for traits influenced by Perennial alleles, while in the HV population trasgressive genotypes were found for traits controlled by only Vania (Internode length). Presumably this was a consequence of the only partial identification of the QTLs involved in the genetics of the character. In order to evaluate the goodness of QTL analysis, a trait influenced by alleles from both Yolo Wonder and Criollo de Morelos: i.e. the pedicel length was analysed (Figure 5). The genotypes of the 3 individuals with the highest ( $\mathrm{A}, \mathrm{B}, \mathrm{C}$ ) and lowest (C, D, E) trait value was assayed using the graphical genotypes approach (Young and Tanksley, 1989). Alleles from Yolo Wonder were found to increase the trait value for QTLs placed on chromosome P1, P4 and LG42; Criollo de Morelos 334 alleles positively influence the trait for QTLs located on chromosome P2, P3 and P12. For the individuals with the highest value, it was found out that their genotype contained all the six alleles increasing the trait value, with the exclusion of the individual B , whose genotype presents the opposite allele on chromosome P12. On the contrary, the individuals with shorter pedicel length contained no one of the alleles responsible for increasing the trait value, with the exclusion of individual D for the QTL located on chromosome P2.

By applying multiallelic breeding programs, favourable allelic combinations may deliver transgressive genotypes which could be exploited for the genetic improvement of important agronomic traits.

| Trait | QTL presents in the F6YC population | QTL presents in the PY population | QTL presents in the HV population |
| :---: | :---: | :---: | :---: |
| Lfw | Lfw3.1 | Lfw3.1 | Lfw3.1 |
|  | Lfw11.1 | Lfw11.1 | Lfw11.1 |
|  | Lfw7.1 | Lfw7.1 |  |
| Frl |  | Frl2.1 | Frl2.1 |
|  | Frl3.1 | Frl3.1 |  |
|  | Frl7. 1 |  | Frl7. 1 |
| Frd | Frd3.1 | Frd3.1 | Frd3.1 |
|  | Frd10.1 | Frd10.1 | Frd10.1 |
|  | Frd11.1 | Frd11.1 | Frd11.1 |
|  |  | Frd2.1 | Frd2.1 |
|  | Frd8. 1 |  | Frd8. 1 |
| Frs | Frs3.1 | Frs3.1 | Frs3.1 |
|  | Frs10.1 | Frs10.1 | Frs10.1 |
|  | Frs11.1 |  | Frs11.1 |
| Flw | Flw2.1 | Flw2.1 | Flw2.1 |
| AxI | Ax12.1 | Ax12.1 | Ax12.1 |
| Nle |  | Nle2.1 | N 2.1 |
| Inl | In11.1 |  | InI1.1 |
|  | Inl2.1 | In2.1 |  |
| Axs | Axs2.2(Yolo) | Axis2.1 | Axs2.1 |

Table 13: QTLs in common to 2 to 3 populations are listed. For each trait, common QTLs are presented.

|  | Population |  |  |  |
| :--- | :--- | :--- | :---: | :---: |
| Trait | Trait | YC | PY | HV |
|  |  |  |  |  |
| Logarith of fruit weight | Lfw | Y | Y | V |
| Fruit length | Frl | YC | PY | HV |
| Fruit diameter | Frd | Y | Y | V |
| Fruit shape | Frs | YC | PY | H |
| Pericarp thickness | Pet | Y | na | na |
| Number of loges | Nlo | YC | na | na |
| Pedidel lenght | Pel | YC | na | HV |
| Flowering earliness | Flw | YC | P | HV |
| Axis lenght | Axl | YC | P | H |
| Number of leaves | Nle | YC | PY | H |
| Internode length | Inl | YC | P | H |
| Internode growth time | Int | YC | PY | V |
| Axis growth speed | Axs | YC | P | H |

Table 12: Parental alleles influencing the horticultural traits analysed in the three progenies. Letters indicate Yolo Wonder (Y), Criolo de Morelos 334 (C), Perennial (P), Vania (V), H3 (H), while na stands for "not available".

## Potential orthologous QTLs with other solanaceae

 speciesSeveral QTLs analyses concerning horticultural traits in Solanaceae species are available, including pepper (Ben Chaim et al. 2001a, Rao et al. 2003), tomato (Bernacchi et al. 1998, Grandillo et al. 1999, Frary et al. 2002), eggplant (Doganlar et al. 2002, Frary et al. 2003) and potato (Van Eck et al. 1994).

In eggplant Doganlar et al. (2002) performed analyses on 16 horticultural traits related to domestication. Frary et al. (2003) focused their attention on 18 traits affecting leaf, flower, fruit and plant characteristics.
By comparing pepper and eggplant QTLs analysis, some putative orthologous regions have been found. For fruit diameter, Doganlar et al. (2002) detected a QTL on chromosome E11 whose position, by
alignment with the integrated map of pepper (Paran et al. 2004) and the comparison between pepper and tomato performed by Livingstone et al. (1999), allow the identification of a putative orthologous QTL in pepper: i.e. Frd12.1.

The fruit weight QTL Fw2.1 identified in eggplant by Doganlar et al. (2002), in pepper by Ben Chaim et al. (2001a) Rao et al. (2003) and Zygier et al. (2005) and in tomato by Grandillo et al. (1999), could be putative orthologous to Lfw2.1 detected in two of the three population analysed in this work. The fruit weight QTL Fw11.1 detected by Grandillo et al. (1999), Doganlar et al. (2002) and Rao et al. (2003) was identified in all of the three pepper populations considered on chromosome P11. Frl 2.1 detected in the PY and HV population could correspond to the FI2.1 QTL detected by Rao et al. (2003) and to tomato fruit length QTL on chromosome T2 (Lippman and Tanksley 2001). Moreover the QTL Frd4.1 seems to correspond to the Fl4.1 QTL detected by Rao et al. (2003) and to the tomato QTL controlling fruit diameter on chromosome T4 (Lippman and Tanksley 2001).
The QTL Frs3.1 identified in pepper by Ben Chaim et al. (2001) and Rao et al. (2003) and in tomato by Bernacchi et al. (1998), and Fs10.1 identified in pepper by Ben Chaim et al. (2001) and in tomato by Grandillo et al. (1999) could correspond respectively to Frs3.1 and to Frs10.1 detected in the pepper populations in study. On the contrary, the major ovary shape QTL in eggplant is located on chromosome E4 (Doganlar et al 2002). At last the Fs10.1 found in pepper seems to be orthologous to Ro gene controlling the tuber shape in potato (Ben Chaim et al. 2003).
Many minor QTIs controlling number of locules were identified in the YC populations, a result in contrast with those reported in tomato (Lippman and Tanksley, 2001) where one major QTL on T11 and 2 minor QTLs on T2 controlling this trait were found.
QTLs analysis on horticultural traits

Figure 5: chromosomes and the LG42 containing QTLs for the pedicel length trait. The colour black in the chromosomes shows the




Two QTLs controlling the flowering earliness detected by Frary et al. (2003) in eggplant and by de Vicente and Tanksley (1993) and Grandillo and Tanksley (1996) in tomato (Fw1.1 and Fw2.1) were also detected in our work, one in the YC progeny (Flw1.1, which was already found by Rao et al. 2003) and one shared by the three populations (Flw2.1), allowing the localisation of important genomic regions affecting the earliness of plants flowering on chromosome P1 and P2. For the primary stem height, a major QTL was always found on chromosome P2. A putative orthologous QTL was also detected by Paran (1997) on tomato and in eggplant by Frary et al. (2003). Results showed that some domesticated-related traits, as the fruit weight and the fruit shape have been conserved during the evolution and the domestication in the Solanaceae family. Notwithstanding, when exploring synteny, orthologous QTLs show differences in effect between species, such as the fs10.1/ovs 4.1 which is the major effect QTL in tomato and eggplant but not in pepper.

## Conclusions

QTL analyses performed using three different intra-specific progenies allowed to more precisely localise and confirm the genomic regions involved in the control of important key traits, such as fruit weight, fruit diameter or fruit shape. The results presented in this chapter show that 35 of the 78 QTLs detected in the YC population and influencing the 13 horticultural traits analysed were found to control the same trait and were located on the same chromosome in the 3 pepper intra-specific maps. Unfortunately, QTLs comparison among pepper tomato and eggplant was limited by the low number of common horticultural traits analysed, and by the limitation in genome alignment due to the lack of shared polymorphic markers.
QTLs analysis on horticultural traits

QTLs analysis on horticultural traits



QTLs analysis on horticultural traits




QTLs analysis on horticultural traits


QTLs analysis on horticultural traits

Figure 6: Synthesis of the QTLs detected for all traits in the 3 progenies. Detected QTLs and their position on each chromosome and LG is shown. The length of the vertical bars represent the confidence interval of the QTLs defined as the LODmax - 1 interval. Traits are indicated with three letters as follows: Logarithm of fruit weight (Lfw), Fruit length (Frl), Fruit diameter (Frd), Fruit shape (Frs), Pericarp thickness (Pet), Number of locules (Nlo), Pedicel length (Pel))Flowering
(Nle), Internode length (Int), Internode growth time (Int), Axis growth speed (Axs).

## Conclusions and perspectives

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The main goal of the present PhD thesis was to integrate genetic information for horticultural traits and disease resistances on a single high resolution linkage map and to generate new tools for further improvements of pepper genome in multitrait breeding programs.

## 1 Conclusion of the experimental work

The experimental activities carried out can be summarized as follows:

- to develop new EST derived SSR markers, to be used for genetic mapping and phylogenetic studies.
- to construct a new high resolution genetic linkage map based on 297 RILs obtained from the cross 'Yolo Wonder' x 'Criollo de Morelos 334' (CM334) and to evaluate the efficiency of mapping and QTL detection in selected subsets of the RIL population.
- to use the newly developed map for identifying QTLs controlling horticultural traits, and compare their position between pepper varieties.
- to analyse macro-synteny among Solanaceae species by comparing the position of QTLs for horticultural traits in pepper, tomato, potato and eggplant.


### 1.1 Development of new SSRs and pepper map improvement

On the whole, 204 pepper EST-derived SSRs were developed and 49 were found to be polymorphic among the parents of the present and/or previously developed pepper maps. Forty-seven SSRs were found polymorphic between the accessions 'Tabasco' and 'P4' (Sasvári et al. 2004), while 21 were successfully mapped in the intra-specific population developed in this study.

The number of alleles detected per SSR marker varied from 2 to 9 (mean 4.5). The largest variation in amplicon size (261-349bp) was present at EPMS-923. The PIC value of each of the 49 mappable markers ranged from 0.24 to 0.86 (mean $0.62 \pm 0.02$ ). EPMS-650 had the highest PIC, and EPMS-603 and EPMS-670 the lowest.
The EST-derived SSR markers were also investigated for their usefulness in diversity analyses by assessing genetic similarity (GS) among 16 test genotypes from eight Capsicum species. Results showed that GS varied from 0.09 ('Tabasco’ vs. ‘CM334') to 0.81 ('Chi1' vs. 'Chi7'). Genetic relationships between the accessions was in good agreement with known taxonomic classifications and with those previously generated by applying RFLP, RAPD and AFLP markers (Prince et al. 1994; Rodriguez et al. 1999; Portis et al. 2006). Thus C. annuum types were clustered together, and two sub-clusters corresponding to fruit morphology characteristics e.g: small fruited types and large-fruited ones, could be recognised as previously reported. The cluster closest to the annuum group contained both frutescens and chinense types, while a less genetically related cluster contained the remaining wild Capsicum species baccatum (vars. baccatum and pendulum), praetermissum, chacoense, eximium and pubescens.
The high level of inter-specific SSR transferability achieved across the eight Capsicum species included in the present study mirrors that experienced in other plant contexts (reviewed by Varshney et al. 2002), which have confirmed that EST-SSRs are highly conserved across species of the same genus (Scott et al. 2000). Some authors pointed out that certain microsatellite PCR primer pairs designed for one organism may not be applicable across genera, because of high variation in flanking sequences of microsatellites between the donor and the receptor, and the frequent loss of the microsatellite motif where amplification occurs (Smulders et al. 1997) as shown between potato
and tomato (Milboune et al. 1998). However, recent results (I. Nagy pers. communication) showed that SSR markers are cross-species transferable in the Solanaceae family as previously demonstrated by Provan et al (1996) and Smulders et al. (1997). On the whole, the present results confirmed that the newly-derived EST-SSRs are informative for phylogenetic and mapping studies; furthermore, they are present within the transcriptome and may represent a valuable tool for mapping genes of known function.

The intra-specific genetic linkage map developed in this thesis may be considered as the new reference map for the following reasons:
i) it has a good level of resolution, both because the population is larger than never used in pepper, and because the use of an F5 generation allowed more meioses to be involved than in the previous F2 or doubled haploid populations used for mapping;
ii) the use of an intra-specific progeny makes it possible to reduce the frequency of skewed segregation, thus increasing mapping precision;
iii) the overall length of the map is 1857 cM , a value analogous to the one of 1832 cM reported for the integrated map developed by Paran et al. (2004), but significantly greater than the one of 1466 cM based on RILs and recently published by Ogundiwin et al. (2005);
iv) the map contains mainly AFLP markers, but also a few SSR and RFLP markers, which are valuable as anchor markers. Although the few RFLP markers did not permit a precise alignment with tomato maps, SSR markers will promote alignment with other pepper maps due to their high level of
inter-specific transferability as previously demonstrated in other plants (Scott et al. 2000, Varshney et al. 2002)
v) the stringent threshold applied (LOD $>8$ and $\mathrm{r}<0.1$ ) to declare linkage groups consistently increased the confidence in marker position and relative order;
vi) because of the high percentage of homozygosity ( $93.75 \%$ theoretically and an observed average of $92.43 \% \pm 3.76 \%$ ), the developed RIL population is immortalised and makes it possible the replication of phenotypic assays across different environments; thus it will supply robust estimates of trait means making the identification of quantitative trait loci more efficient and reliable.

The use of stringent threshold in declaring linkage groups generated 49 LG of which 23 were successfully assigned to the 12 haploid pepper chromosomes, while 26 remain still unassigned. Maps comparison was possible thanks to 32 shared markers, which allowed the assignment of 23 LGs with regions on the pre-existing maps. All common AFLP markers mapped at the same location, thus confirming their locusspecificity (Rouppe van der Voort et al. 1997; Lefebvre et al. 2002). The alignment was successful for all the chromosomes except P1 and P8 from interspecific crosses, as previously reported in chapter 2.

### 1.2 GENOMIC ORGANIZATION OF QTLS FOR HORTICULTURAL TRAITS

In pepper, QTL analyses were previously mainly focused on genetics of resistance to biotic stresses (Grube et al. 2000b, djian Caporalino et al 2006). QTL analyses of horticultural traits (fruits and plant phenotype) was restricted to a few crosses and plant traits were poorly considered (Ben Chaim et al. 2001; Rao et al. 2003, Zygier et al.
2005). In the present study genomic regions controlling both fruit and plant traits were analysed in 3 distinct crosses involving 5 parental lines, which were previously used for the analysis of multiple disease resistance. The 78 QTLs detected in the Yolo Wonder x CM334 population were compared with those identified in two other populations PY (Perennial x Yolo Wonder) and HV (H3 x Vanai). Most of the QTLs were shown to be clustered on chromosomes P2, P3, P10 and P11 for fruit traits, and P2 for plant traits. These QTL clusters were shared among the 3 progenies. Previous analyses in intraspecific and interspecific pepper progenies also delivered clusters of QTLs for fruit traits on the same chromosomes (Ben Chaim et al. 2001; Rao et al. 2003, Zygier et al. 2005), indicating a large conservation of allele position within the Capsicum genus. On the other hand, parent-specific QTLs were also detected in other chromosomes for fruit as well as plant traits indicating that diversity is available within the $C$. annuum species and that distinct alleles can be exploited in different crosses.

Several common QTLs were found to be shared among the three populations. By comparing the results of QTLs analyses performed in this thesis with those previously carried out in other Solanaceae genera (Grandillo et al. 1999, Ben Chaim et al. 2001, Doganlar et al. 2002, Rao et al. 2003 Zygier et al. 2005) several putative orthologous regions have been identified, in particular for traits referred to the fruit weight, fruit diameter fruit length, fruit shape, flowering earliness and plant height.

The present results show that only $10 \%$ of the QTLs detected in the YC population have putative correspondent QTLs in both tomato and eggplant, a value in contrast to the $40 \%$ of loci detected by Doganlar et al. (2002). Unfortunately, QTLs comparison among pepper tomato and eggplant was hampered by the limited number of shared markers between these genera, and particularly between our map and the tomato, potato and eggplant maps, preventing any conclusion toward
orthology. A further constrain was due to the low number of common horticultural traits analysed, which limited the identification of the chromosomic regions involved in traits control. On the whole, QTL analyses performed using three different intra-specific progenies allowed a more precise localisation of the genomic regions involved in the control of important key traits, such as fruit weight, fruit diameter and fruit shape.

A further activity carried out in the present PhD thesis was to identify sub-samples of individuals suitable for both mapping and QTL analyses. This was achieved by applying the MapPop software from Brown and Vision (2000). Four subpopulations of 141, 93, 45 or 29 individuals were identified and their potential for mapping was tested. Results showed that larger subpopulations delivered reasonable mapping precision, which as expected was higher when the largest subpopulation was genotyped. Moreover when the attention was focused on the minimum subset of individuals which allowed to assigned a new marker to a linkage group, the 45 individual subpopulation made it possible to obtain reliable results. The quality of QTL detection was assayed on three traits, by comparing the results obtained in the RIL population of 297 individuals as well as in the subpopulations of 141 and 93 individuals. This approach was useful for traits controlled by major QTLs with an $R^{2}$ value $>30-40 \%$. On the contrary, the QTLs with weak effect controlling plant height were detectable only in the whole population. This clearly shows that polygenic traits governed by multiple genes with weak effects require large mapping populations for exhaustive genetic analyses.

## 2 Perspectives

The results here reported open new questions, and suggest to prosecute experimental work as reported below.

### 2.1 Map improvement

The map developed during the PhD thesis will require further improvement. The saturation of the YC map but also a better anchorage with the previously developed pepper maps are needed.
Several linkage groups still remain unassigned, thus further markers has to be applied for the detection of bridge markers which will help to merge these unlinked LGs and to attribute them to chromosomes. AFLP markers represent an useful tool as they generate many polymorphic fragments per primer combination, thus increasing the probability of detecting useful bridge markers. However a limiting factor is due to their trend to cluster and not uniform distribution in the genome. Suitable classes of markers are RFLPs, SSRs and SNPs, which are frequently co-dominant, and more informative. The main limiting factor in using RFLPs and SSRs is their low polymorphism within C. annuum, which makes necessary to develop and test many of them for obtaining a low number of useful markers. SNPs, as well, are present in a high number throughout the plant genomes and might also be used for their linkage to functional genes. However, their development is costly and time consuming and this may hamper their use. A possible alternative to the use of new SNPs might arise by the application of RFLPs, COSII and SNPs already mapped in other Solanaceae species. These might also allow to compare different pepper maps with the aim of highlighting the occurrence of reciprocal chromosomal translocations which have taken place in Capsicum speciation. These translocations might be responsible for pseudolinkage phenomenon between loci close to the
interchange breakpoint, in particular for chromosome P1 and P8. The use of telomeric markers will be useful to both saturate and more precisely define the sizes of pepper chromosomes.
New markers might be selected by using the subpopulation of 45 individuals identified by applying the MapPop software (Brown and Vision, 2000) thus reducing time and costs for future genotyping.
In addition a new integrated map will be developed by merging the three intra-specific maps developed (YC, HV and PY). The new integrated map could bring to an improvement in the QTL analysis, allowing to better detect QTLs position on chromosomes.

### 2.2 Widen the knowledge of the genetic basis controlling horticultural traits and pathogen resistance .

The QTL analyses performed on 13 horticultural traits contributed to a better understanding of pepper genome organization. New horticultural traits related to plant architecture and development has been analysed. The integration of these new information with those related to the genetic determinism of resistance to pepper diseases will make it possible to better identify useful markers and suitable strategies for future application of breeding programs based on Marker Assisted Selection (MAS). Information at present available on genes and QTLs for horticultural and disease resistance traits highlight the following:

- In chromosome P1, P2, P3, P4, P10, P11 and P12 linkages between fruit traits, plant traits and disease resistance traits have been identified (Figure 1). The accumulation of resistance alleles to several pathogens (Phythophthora, powdery mildew, potyviruses, CMV, TSWV) and genetic components linked to large fruit or
early-vigorous plants will require multiple recombination in the targeted chromosomes
- Chromosomes P6 and P7 also cumulate QTLs for both resistance and qualitative traits, but larger genetic distance may favour selection of recombinant individuals.
- Chromosome P5 includes only disease resistance QTLs (Phytophthora, CMV, powdery mildew)
- Chromosome P8 contains only QTLs for horticultural traits.
- In chromosome P9 the imprecise location of Me (nematode resistance) genes needs further linkage analysis to better target the relative location of Me-genes cluster and horticultural trait QTLs.

To better precise QTLs location, gene candidate approach and synteny among species will be used. In particular the genetic information from Arabidopsis thaliana and other plant species will be used to generate markers located near putative genes of interest which might be mapped.

### 2.3 Towards the MAS (Marker Assisted Selection)

Breeding programs based on MAS strategies represent the applicative extension after the high resolution mapping and QTL analyses performed in this study. Previous experience in MAS selection for polygenic traits demonstrated that MAS is effective in selecting the targeted QTLs (foreground selection) and accelerating the recovering of the expected genetic background (background selection) (Hospital et al 1997). However, unfavourable genetic linkages may hamper the side by side progress for both horticultural and resistance traits(Thabuis et al 2004 a and b).

Selection for multiple traits requires first of all to cross parental lines (biparental or multiparental crosses) with the goal of including the totality of favourable alleles (Ribaut and Betran, 1999) and to select the expected recombinant individuals in the progenies. The selection of large fruited cultivars resistant to pathogens will depends on the genetic structure of chromosomes carrying QTLs. In the RILs population developed, chromosomes P4, P5, P6, P10, P11, P12 show that P. Capsici resistant alleles are conserved between parental lines together with plant and/or fruit traits QTLs.

On the other hand, in chromosome P9, the nematode resistance genes are linked in repulsion to fruit traits QTLs.

Graphical genotype of the RIL individuals will have to be examined to select individuals with interesting recombinations which will be used as parental lines of future breeding programs.
By using the multiparental approach, markers have to be evaluate for their polymorhism and some markers may have to be converted from RFLP or AFLP into SCARs, STSs, SNPs to obtain reliable and codominant markers which are less time-consuming and less costly to assay. These markers might also be transferable to related species, thus increasing the possibility of detecting important genomic regions controlling for traits of interest of Solanaceae species.
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 Figure 1: Maps aligned from the three populations analysed. QTLs position for horticultural and resistance traits to resistance genes previously mapped are showed for each chromosome.

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The objective of the present PhD thesis was to develop a high resolution intra-specific map of pepper (C. annuum L.) in order to integrate genetic information for horticultural traits and to generate new tools for further improvements of pepper genome in multitrait breeding programs.

At first 49 EST derived SSR markers were developed for mapping purposes, and for providing anchor markers in crossspecies comparative mapping.

The high resolution intra-specific map was constructed using a population of 297 F5 recombinant inbred lines. The parents were the large-fruited inbred cultivar 'Yolo Wonder' and the hot pepper line 'Criollo de Morelos 334'. A total of 597 molecular markers were applied. The framework map covered 1857 cM with an average inter-marker distance of 5.71 cM . In addition the use of the largest population ever used in pepper and he use of an F5 generation provided a good level of resolution.

A QTL analysis of 13 horticultural traits (i.e fruit and plant traits) was performed in the RIL mapping population as well as in two double haploid populations originating from the crosses Perennial X Yolo Wonder and H3 X Vania. A total of 14 and 6 common QTLs controlling fruit and plant traits respectively were found to be shared among the three populations.
An analysis of synteny among tomato, pepper, potato and eggplant for common genomic regions controlling horticultural traits was carried out. Putative orthologous regions were found, which may have been conserved during evolution and independent selection performed on these species.

Through selective genotyping subsets of the most informative RILs (141, 93, 45 or 29) were identified, in order to reduce the genotyping and phenotyping efforts. Results showed that larger subpopulations delivered reasonable mapping precision. On the contrary, the selective genotyping approach for QTL detection seems not appropriate because only major QTLs with an $R^{2}$ value $>30-40 \%$ were detected in the RIL subsets.

L'objective de la thèse était de développer une carte intraspecifique a haute résolution du piment (C. annuum. L) pour intégrer l'information génétique de caractères horticulturaux et pour générer des outils pour la sélection multi-caractères chez le piment.

La carte a haute résolution a été construite en utilisant une descendance recombinante de 297 lignées F5. Les parents utilisés étaient la variété commerciale Yolo Wonder et la variété épicé Criollo de Morellos 334. Un totale de 597 marqueurs moléculaires (ont été utilisés. La carte trame couvrait 1857 cM avec une distance moyenne entre marqueurs de $5,71 \mathrm{cM}$. Le bon niveau de résolution dérivait soit de la taille de la population utilisés, soit de l'utilisation d'une génération F5.

Une analyse QTL sur 13 caractères horticulturaux liés a caractéristiques des fruits et de la plante a été réalisée, en utilisant soit la populations de cartographie soit deux populations haploïdes doublées dérivées des croisements Perennial X Yolo Wonder et H3 X Vania. Un total de 14 et 6

QTL communes ont été détectées entre les 3 populations en étude.

Une analyse de synténie entre tomate, piment pomme de terre et aubergine a été réalisée pour les régions génomiques contrôlant les caractères horticulturaux. Plusieurs régions conservées ont été découvertes, que peuvent avoir été conservées pendant l'évolution et la sélection de ces espèces.

En utilisant le «selective genotyping", sous groupes des individus les plus informatives (141, 93, 45 ou 29) ont été identifiés pour réduire les efforts du marquage et du phénotypage. Les résultats démontraient que les souspopulations les plus grandes permettaient de garantir une bonne résolution en cartographie. Au contraire le "selective genotyping" ne ressemblait pas utile pour l'analyse QTL car seulement les QTL a effet fort et avec une valeur de $R^{2}>30-$ $40 \%$ ont été détectées dans les sous-groups considérés.


[^0]:    Pepper intra-specific genetic linkage map - Carte du piment intra spécifique

[^1]:    Figure 2. Framework map consisting of 323 markers, obtained from the F5YC RIL mapping population. In normal bold, markers used as anchors to assign LGs to chromosomes. Markers on the right of the chromosomes are unordered but placed by the "try" command. In Italic and bold, SSR markers. \# indicates markers showing segregation distortion at P<0.01 in the framework map. Chromosome orientation follows Lefebvre et al. (2002).

